Volume 44 *June 1958* *Number 6*

Proceedings of the
National Academy of Sciences
of the United States of America

The Proceedings of the National Academy of Sciences

OFFICERS OF THE ACADEMY

DETLEV W. BRONK
President

FARRINGTON DANIELS
Vice President

JOHN G. KIRKWOOD
Foreign Secretary

HUGH L. DRYDEN
Home Secretary

WILLIAM J. ROBBINS
Treasurer

EDITORIAL BOARD OF THE PROCEEDINGS

WENDELL M. STANLEY
Chairman

HUGH L. DRYDEN, *Home Secretary* — JOHN G. KIRKWOOD, *Foreign Secretary*
WILLIAM J. ROBBINS, *Treasurer* — EDWIN B. WILSON, *Managing Editor*

J. W. BEAMS	G. W. BEADLE	GREGORY BREIT
R. E. CLELAND	E. A. DOISY	L. C. DUNN
W. W. RUBEY	G. C. EVANS	W. F. LIBBY
HARLOW SHAPLEY	C. H. GRAHAM	J. VON NEUMANN
F. E. TERMAN	J. T. PATTERSON	PAUL WEISS

Subscription price is $12.50 for 1 year, $24.00 for 2 years, and $34.50 for 3 years; the price of a single issue is $1.50. Make all remittances payable to The University of Chicago Press in U.S. currency or its equivalent. Authorized agent for the British Commonwealth, except North America and Australasia: Cambridge University Press, Bentley House, 200 Euston Road, London, N. W. 1, England.

Editorial correspondence should be addressed to the PROCEEDINGS of the NATIONAL ACADEMY OF SCIENCES, Attention Miss Mary D. Alexander, University of Chicago Press, 5750 Ellis Avenue, Chicago 37, Illinois.

Business correspondence should be addressed to the Journals Division, University of Chicago Press, 5750 Ellis Avenue, Chicago 37, Illinois.

Subscribers are requested to notify The University of Chicago Press and their local postmaster immediately of change of address. Notices to the Press should provide both the old and the new address.

Microfilms of complete volumes of this journal are available to regular subscribers only and may be obtained at the end of the volume year from University Microfilms, 313 N. First Street, Ann Arbor, Michigan.

Entered as second-class matter June 21, 1920 at the Post-office at Easton, Pennsylvania, under the Act of August 24, 1912. Accepted for mailing at a special rate of postage provided for in Section 1103, Act of October 3, 1917. Authorized June 18, 1920.

PRINTED IN THE U. S. A.

THE PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES
is published for
THE NATIONAL ACADEMY OF SCIENCES
by
THE UNIVERSITY OF CHICAGO PRESS

Proceedings of the
NATIONAL ACADEMY OF SCIENCES

Volume 44 · Number 6 · June 15, 1958

A THEOREM ON FORCE-FREE MAGNETIC FIELDS

By L. Woltjer

UNIVERSITY OF CHICAGO

Communicated by S. Chandrasekhar, May 5, 1958

Chandrasekhar and Woltjer[1] have shown that the force-free magnetic fields with "constant α" can be related to a variational principle. Here α is the scalar function in the equation

$$\text{curl } \boldsymbol{H} = \alpha \boldsymbol{H}, \tag{1}$$

which characterizes the force-free fields. This equation follows from the definition of force-free magnetic fields as fields in which the Lorentz force vanishes.

It was shown that the force-free fields with constant α are among the fields with maximum magnetic energy for a given mean square current density. Alternatively, this was formulated by stating that these fields are among the fields of minimum dissipation for a given magnetic energy.

There are several reasons why the above-mentioned formulations are not entirely satisfactory. It is not obvious physically why we should consider the mean square current density as given. Moreover, these formulations do not lead us immediately to force-free fields but to a wider class of fields, which satisfy the equation

$$\text{curl curl } \boldsymbol{H} = \alpha^2 \boldsymbol{H}; \tag{2}$$

and additional arguments are then needed to arrive at equation (1). Difficulties also arise because of the presence of surface currents.

In this paper we shall prove another variational principle which states that the force-free fields with constant α represent the state of lowest magnetic energy in a closed system. A principle of this kind seems intuitively obvious, because, if the magnetic energy is a minimum, the field can produce no motions, and therefore the Lorentz force must vanish. The proof of the principle consists in two parts: We first show that in a closed system

$$\int_V \boldsymbol{A} \cdot \text{curl } \boldsymbol{A} dV = \text{constant}, \tag{3}$$

if the gauge of the vector potential $\boldsymbol{A}$ is suitably chosen. Next we show that, if we minimize the magnetic energy while satisfying equation (3), we obtain the force-free condition with a constant α.

If the conductivity is infinite, the induction equation is

$$\frac{\partial \boldsymbol{H}}{\partial t} = \text{curl}\,(\boldsymbol{v} \times \boldsymbol{H}). \tag{4}$$

where $\boldsymbol{v}$ is the fluid velocity. Introducing the vector potential and choosing the gauge so that the scalar potential vanishes, this becomes

$$\frac{\partial \boldsymbol{A}}{\partial t} = \boldsymbol{v} \times \text{curl}\,\boldsymbol{A}. \tag{5}$$

Therefore

$$\text{curl}\,\boldsymbol{A} \cdot \frac{\partial \boldsymbol{A}}{\partial t} = 0. \tag{6}$$

Making use of this relation, we obtain, on integrating by parts,

$$\frac{\partial}{\partial t}\int_V \boldsymbol{A} \cdot \text{curl}\,\boldsymbol{A} dV = \int_V \boldsymbol{A} \cdot \text{curl}\,\frac{\partial \boldsymbol{A}}{\partial t}\, dV$$

$$= \int_V \text{curl}\,\boldsymbol{A} \cdot \frac{\partial \boldsymbol{A}}{\partial t}\, dV + \int_S \boldsymbol{A} \times \frac{\partial \boldsymbol{A}}{\partial t}\, dS = 0. \tag{7}$$

The surface integral vanishes because we consider a closed system. For then the motions inside the system may not affect the vector potential outside, and, as the vector potential is continuous, even when surface currents are present, $\partial \boldsymbol{A}/\partial t$ must vanish at the surface of the system.

We now ask for the minimum of the magnetic energy,

$$\frac{1}{8\pi}\int_V |\text{curl}\,\boldsymbol{A}|^2\, dV, \tag{8}$$

subject to condition (3). Making use of a Lagrangian multiplier α, we find that this requires

$$\int_V [2\,\text{curl}\,\boldsymbol{A} \cdot \text{curl}\,\delta\boldsymbol{A} - \alpha(\delta\boldsymbol{A} \cdot \text{curl}\,\boldsymbol{A} + \boldsymbol{A} \cdot \text{curl}\,\delta\boldsymbol{A}]dV = 0. \tag{9}$$

Integrating by parts and observing that $\delta\boldsymbol{A}$ must vanish at the surface for the reasons we have already given, we obtain

$$\int_V [\text{curl curl}\,\boldsymbol{A} - \alpha\,\text{curl}\,\boldsymbol{A}] \cdot \delta\boldsymbol{A} dV = 0. \tag{10}$$

Since $\delta\boldsymbol{A}$ is arbitrary, the integrand must vanish identically, and we must have

$$\text{curl curl}\,\boldsymbol{A} - \alpha\,\text{curl}\,\boldsymbol{A} = 0. \tag{11}$$

Because of the definition of the vector potential, this is equivalent to

$$\text{curl}\,\boldsymbol{H} = \alpha\boldsymbol{H}. \tag{12}$$

Thus the force-free fields with constant α represent the lowest state of magnetic energy which a closed system may attain.

This has two important consequences. It proves in a general way the stability

of force-free fields with constant α. Under restricted circumstances the stability of force-free fields with constant α has been demonstrated elsewhere more explicitly.[2] It also shows that in a system in which the magnetic forces are dominant and in which there is a mechanism to dissipate the fluid motions the force-free fields *with constant* α are the natural end configurations. The presence and stability of force-free fields when α is not a constant are not excluded by the present arguments, although they appear somewhat unlikely, as they are not stable against large perturbations.

Some mechanism is needed to dissipate the velocities. In astrophysical cases the viscosity is too small to be effective. But it seems not improbable that sufficient dissipation may be obtained by the Fermi mechanism of the acceleration of cosmic-ray particles. Similar suggestions have already been made for the dissipation of motions in the Crab nebula[3] and in interstellar space.[4] The present discussion is relevant if the conductivity is sufficiently large, i.e., if the time of decay of the magnetic field is longer than the damping time for the motions. This condition will probably be fulfilled in most astrophysical situations.

It is a pleasure to acknowledge many valuable discussions on force-free fields with Professor S. Chandrasekhar.

Note added in proof. Since this paper was written we have found that the hydromagnetic equations have other integrals besides the integral (3). The very general equilibrium configurations which may be derived from these integrals will be discussed in a forthcoming paper.

[1] S. Chandrasekhar and L. Woltjer, "On Force-Free Magnetic Fields," these PROCEEDINGS, **44**, 285, 1958.

[2] L. Woltjer, "The Stability of Force-Free Magnetic Fields," *Ap. J.* (in press).

[3] L. Woltjer, "The Crab Nebula," *B.A.N.*, **14**, 39 (No. 483), 1958.

[4] E. N. Parker, "Origin and Dynamics of Cosmic Radiation," *Phys. Rev.*, **109**, 1328, 1958.

---

*THE EFFECTS OF HYDROXYLAMINE ON THE $C^{14}O_2$ FIXATION PATTERN DURING PHOTOSYNTHESIS**

BY J. A. BASSHAM, M. KIRK, AND M. CALVIN

RADIATION LABORATORY AND DEPARTMENT OF CHEMISTRY, UNIVERSITY OF CALIFORNIA, BERKELEY, CALIFORNIA

Communicated March 25, 1958

Evidence for an unstable product or products formed in the early stages of carbon fixation during photosynthesis by algae has been presented by Metzner *et al.*[1] In an attempt to stabilize, isolate, and characterize such unstable products, Metzner *et al.*[2] added hydroxylamine to the algae just before, during, or after a short period of photosynthesis with $C^{14}O_2$.

Analysis of the products of such fixation by the usual techniques of paper chromatography and radioautography led to observation of an apparently new radioactive compound on the chromatogram. This compound or "spot" usually appeared in the two-dimensional papers as a thin streak (its long dimension in the phenol direction) somewhere between aspartic and malic acids. Observations that the spot

appeared to coincide with one of two fluorescent streaks (sometimes there was a radioactive compound coinciding with each of them) and that it might have an ultraviolet absorption peak of 285 mμ led to the hypothesis that the new compound might be a complex of newly incorporated $C^{14}O_2$, possibly including a pterine.

We have attempted to identify this substance and to elucidate its role in photosynthesis. We have frequently obtained enhancement of C^{14} fixation by the addition of hydroxylamine hydrochloride to the photosynthesizing algae (both *Chlorella* and *Scenedesmus*) and have observed the fluorescent spots and a new radioactive spot (hereafter called *F'*) which we believe to be the same as those previously reported, at least in the two-dimensional chromatograms. Our results may be summarized as follows:

1. Addition of hydroxylamine hydrochloride to a concentration of 0.1 *M* in the suspension of photosynthesizing algae just before or during their fixation of $C^{14}O_2$ for 5 seconds in the light usually stimulates the incorporation of the C^{14} into compounds stable to our usual techniques (paper chromatography, etc.). The increase in radioactivity is seen in the phosphate esters, particularly phosphoglyceric acid.

2. A similar increase in fixation of C^{14} is seen if hydroxylamine hydrochloride is added to the algae suspension prior to or during dark fixation of $C^{14}O_2$ immediately following a period of illumination.

3. Addition of hydrochloric acid alone in an amount producing the same pH (4.8) in the algae suspension resulted in as much stimulation in one experiment as did the addition of hydroxylamine hydrochloride. In the same series of experiments, addition of neutralized hydroxylamine caused as inhibition of $C^{14}O_2$ uptake.

4. Addition of hydroxylamine or hydroxylamine hydrochloride with the alcohol at the time of killing sometimes results in a slight increase in fixation and other times in no increase at all. However, if hydroxylamine hydrochloride is added for as short a time as 1 second before the algae are killed, there is sometimes a definite increase (around 10 per cent higher than in the controls) in $C^{14}O_2$ fixation.

5. In cases 1 and 2 above, there appears to be a decrease in C^{14} incorporated into malic acid.

6. In cases 1, 2, and 4 above, the radioactive spot or streak (*F'*) formed in the presence of hydroxylamine and running between aspartic acid and malic acid *often, but not always*, coincides with one or the other of the fluorescent spots which run in the same area. Owing to the fact that the radioactive compound and the fluorescent compound do not coincide in every case, it appears that they are not the same substance and that their frequent coincidence is a chromatographic accident, caused perhaps by their both being pushed along in the butanol–propionic acid direction by a third substance, which may be hydroxylamine salt itself.

7. The radioactive spot, on re-chromatography in the same solvents, gives a spot which is phosphoenolpyruvic acid. This identity has been established by co-chromatography with phosphoenolpyruvic acid (from California Foundation for Biochemical Research) followed by spraying with the Haynes-Isherwood phosphate spray. It was also established by co-chromatography (with pyruvic acid) of the radioactive compound obtained after treating the original spot with phosphatase. The position of the pyruvic acid on the paper was located by treatment with silver nitrate in acetone, followed by spraying with ethanolic sodium hydroxide.

8. When the radioactive spot, identified as phosphoenolpyruvic acid and ob-

tained from a photosynthesis experiment with $C^{14}O_2$, is treated with 0.01 *M* hydroxylamine hydrochloride at room temperature for 1 hour and the resulting solution is concentrated and chromatographed, a radioactive spot is obtained which has the same appearance and location on the chromatogram as the spot *F'*.

These results lead to the following conclusions:

1. The stimulation of fixation of $C^{14}O_2$ by hydroxylamine, which is most noticeable at very short exposures of the algae to C^{14}, is an actual acceleration of one or more steps of the photosynthetic incorporation of CO_2 rather than the stabilization of an unstable intermediate. The entry of $C^{14}O_2$ into the cell seems a likely place at which stimulation might take place.

2. Hydroxylamine may form a compound or a stable salt with phosphoenolpyruvate, and this compound is stable only in the presence of excess hydroxylamine. Alternatively, hydroxylamine affects the chromatographic behavior of phosphoenolpyruvate in some manner to cause the observed movement on the chromatogram found experimentally. Once the excess of hydroxylamine is removed (rechromatography), phosphoenolpyruvate runs to its usual position on the chromatogram.

3. The decrease in radioactivity found in malic acid may indicate that the hydroxylamine reacts with phosphoenolpyruvic acid in the algae during the period of photosynthesis and thereby inhibits C_1 to C_3 carboxylation.

4. The fluorescence which often coincides with the radioactive spot is due to a different substance than the radioactive spot, and the coincidence is accidental.

While it thus appears that the effect of hydroxylamine is not a stabilization of unstable compounds, as was suspected earlier, the concept of a rather unstable CO_2 complex which could serve as a C_1 donor in the carboxylation reaction of the photosynthetic carbon reduction cycle is not ruled out by this fact. However, the principal instance in which there has appeared to be more total radiocarbon fixed during a short period of photosynthesis than is normally seen by the usual killing and analytical procedures (e.g., hot ethanol extraction and two-dimensional chromatography) was in the experiments in which hydroxylamine hydrochloride was added just prior to killing. Thus the hypothesis of an unstable CO_2 bearing intermediate (other than the β-keto acid carboxylation product from ribulose diphosphate) must be considered to be unsubstantiated by direct evidence at the present time.

* The work described in this paper was sponsored in part by the United States Atomic Energy Commission and in part by the Department of Chemistry, University of California, Berkeley, California.

[1] H. Metzner, H. Simon, B. Metzner, and M. Calvin, these PROCEEDINGS, **43**, 892, 1957.

[2] H. Metzner, B. Metzner, and M. Calvin, these PROCEEDINGS, **44**, 205, 1958.

*PROTEIN BIOSYNTHESIS BY A CELL-FREE BACTERIAL SYSTEM**

By Mirko Beljanski† and Severo Ochoa

DEPARTMENT OF BIOCHEMISTRY, NEW YORK UNIVERSITY COLLEGE OF MEDICINE, NEW YORK, NEW YORK

Read before the Academy, April 28, 1958

INTRODUCTION

The mechanism of protein biosynthesis has been extensively investigated during the last few years. Work by Zamecnik and his collaborators[1] has led to the view that, in animal tissues, enzymes present in the cytoplasmic fluid[2] catalyze a carboxyl activation of the amino acids by ATP,[3] followed by transfer of the amino acid residues to RNA and their incorporation into the protein of microsomal ribonucleoprotein particles. This view has been strengthened by recent work from various laboratories.[4,5,6] Amino acid-activating enzymes have also been found in yeast,[7] bacterial,[8,9] and plant[10] cells. There appear to be as many activating enzymes as there are amino acids, and enzymes strictly specific for methionine[7] and tryptophan[11] have been isolated in highly purified form. In the presence of ATP, radioactive pyrophosphate (PP^{32}), Mg^{++}, and amino acid, the activating enzymes catalyze a rapid incorporation or exchange of PP^{32} into ATP, which affords a sensitive method of assay. The reaction has been formulated[2] as in equation (1):

$$\text{ATP} + \text{amino acid} + \text{enzyme} \rightleftharpoons \text{aminoacyl-AMP-enzyme} + \text{PP}. \qquad (1)$$

Because of their high rate of multiplication, bacterial cells should be particularly appropriate for studies of protein biosynthesis and have been used by several investigators.[12,13,14] Gale and Folkes[13] have described a cell-free system from *Staphylococcus aureus* which actively incorporates amino acids into protein and appears to bring about the synthesis of certain enzymes.

We have found, in agreement with Gale, that in *Alicaligenes faecalis* the amino acid–incorporating activity is mainly associated with a particulate fraction, consisting largely of cell membrane fragments, sedimented by centrifugation at relatively low speed (20,000 $\times g$) after disruption of the cells. The same is true of *Azotobacter vinelandii*.[15] It is the purpose of this paper to report on a hitherto unrecognized enzyme which stimulates the incorporation of amino acids into protein of the *Alcaligenes* particles. This enzyme, which is present in the supernatant extract, has been isolated in highly purified form. The purified enzyme does not catalyze the amino acid–dependent exchange of PP^{32} with ATP and this activity also appears to be absent from the particles.

METHODS

Preparation of Particles.—A. faecalis (8750) was grown as described by Pinchot[16] with vigorous shaking for 16 hours at 32°. The cells were harvested by centrifugation and washed twice at 4° with 0.2 *M* potassium chloride. Five to 6 gm. of cells, suspended in 25 ml. of 0.05 *M* Tris buffer, pH 7.0, were disintegrated for 4–8 minutes at 70 volts and about 7° in a 10-Kc Raytheon sonic oscillator. The resulting suspension was centrifuged for 10 minutes at 2000 $\times$ g, to remove intact cells and large cell debris, and the sediment discarded. After repeating this centri-

fugation once more, the supernatant fluid was centrifuged at top speed of the Servall, Model SS1 centrifuge in the cold room for 20 minutes, and the sediment suspended evenly in 15 ml. of ice-cold 0.05 *M* Tris buffer. This suspension was centrifuged once more for 3 minutes at 2000 × *g*, to insure removal of unbroken cells, and the supernatant fluid dialyzed for 1 hour at 4° against 500 ml. of Tris buffer. The particles were then collected by centrifugation in the Servall angle centrifuge as above and resuspended in Tris buffer, either without further treatment (unwashed particles) or after washing three times at room temperature, each with 5 ml. of 1.0 *M* sodium chloride and once with 5 ml. of water (washed particles), to give a suspension containing 12 mg. of particles (dry weight) per ml. Washing of the particles, whether preceded or not by ribonuclease, only reduced their nucleic acid content by 40–50 per cent. Electron micrographs showed that the particles consisted mainly of irregularly shaped cell-membrane, and probably wall, fragments of widely differing sizes. Particle preparations lose about half of their activity if kept overnight at 0°. For this reason, only freshly prepared particles were used in this work.

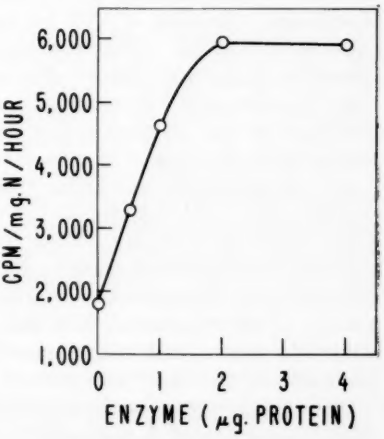


FIG. 1.—Stimulation of glycine incorporation into protein of *Alcaligenes* particles as a function of the concentration of amino acid incorporation enzyme. *Ordinate*, C^{14}-glycine incorporation in cpm per mg. protein nitrogen of particles per hour. The samples contained: Tris buffer, pH 7.0, 50 μmoles; $MgCl_2$, 5.0 μmoles; K_2HPO_4, 5.0 μmoles; C^{14}-glycine (specific radioactivity, 120,000 cpm per μmole), 0.25 μmole; washed particles with 300–400 μg. of protein; and amino acid incorporation enzyme, specific activity (glycine) 24, as indicated. Final volume, 1.0 ml. Incubation in air, 1 hour at 30°.

Measurement of Amino Acid Incorporation.—The composition of the reaction mixtures and incubation conditions were as described for Figure 1. The reaction was stopped by addition of 5 ml. of 10 per cent trichloroacetic acid. After cooling in ice for 10–15 minutes, the precipitate was centrifuged off, washed twice with 5 ml. of 10 per cent trichloroacetic acid, twice with hot (100°) 5 per cent trichloroacetic acid, each time for 15 minutes, and then with acetone. The precipitate was collected by filtration on a filter-paper disk and its radioactivity determined with a thin-window counter. Results are expressed as cpm, or millimicromoles of amino acid, incorporated, per milligram of protein nitrogen of particles (determined by the micro-Kjeldahl method) per hour.

PP^{32}-ATP Exchange Assay.—The assay conditions were as described for Table 2. The reaction was stopped with 2 ml. of 10 per cent trichloroacetic acid and the mixture centrifuged. The precipitate was washed once with 2 ml. of 10 per cent trichloroacetic acid and the supernatant fluid combined with the first. Absorption of the ATP on charcoal, washing, and counting were done essentially as described by DeMoss and Novelli.[8]

RESULTS

Amino Acid Incorporation into Protein of Particles.—All of the (D,L-, or L-) C^{14}-labeled amino acids so far investigated are incorporated to a greater or lesser extent into protein of *Alcaligenes* particles. Some unlabeled D-amino acids (D-threonine, D-leucine) did not depress the incorporation of their labeled L-enantiomorphs, suggesting that they were not utilized by this system, but others (D-glutamic acid, D-phenylalanine) caused a significant decrease. The incorporation is linear with time for acout 1 hour and continues at a decreased rate for another 2 or 3 hours.

There is no incorporation when the incubation is carried out in nitrogen, and that observed in air is inhibited by 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation. Addition of ATP has little effect, presumably because of failure to reach the active sites. Thus, as in the case of isolated nuclei,[17] the system seems to depend on oxidative phosphorylation for generation of the ATP required for activation and subsequent incorporation into protein of the amino acids. It should be mentioned in this connection that the particles exhibit a small oxygen uptake, apparently at the expense of endogenous substrate.

As observed by Gale and Folkes[13] with *Staphyloccocus*, washing of the *Alcaligenes* particles with 1.0 *M* NaCl results in a more or less marked reduction of their amino acid–incorporating activity. Their activity can be increased to levels higher than observed with unwashed particles by addition of the supernatant fluid obtained after removing the particles by centrifugation of the bacterial extract. Most of the activity of the supernatant fluid was thermostable, but a small fraction was destroyed by heating and was presumably enzymatic in nature. A similar thermolabile activity was found in extracts of *A. vinelandii* and *Escherichia coli*.

On assaying enzyme fractions obtained in the course of purification of polynucleotide phosphorylase of *A. vinelandii*[18] for stimulation of amino acid incorporation by washed particles of *A. faecalis*, the two activities (phosphorylase and stimulation of amino acid incorporation) were found to increase together through several purification steps but were eventually separated from each other. Highly purified preparations of the enzyme stimulating amino acid incorporation have now been obtained from *A. faecalis* extracts. These preparations are free of activating enzymes catalyzing the amino acid–dependent exchange of PP^{32} with ATP. Pending elucidation of its nature and mode of action, the new enzyme will be provisionally referred to as the amino acid incorporation enzyme.

Amino Acid Incorporation Enzyme.—The enzyme assay was based on the stimulation of C^{14}-glycine incorporation by NaCl-washed *Alcaligenes* particles. As shown in Figure 1, with highly purified enzyme, this stimulation is proportional to the concentration of enzyme within rather narrow limits, since, as the concentration of incorporation enzyme is increased, a plateau is soon reached. At this point, further increase of the enzyme concentration (up to ten-fold) is without effect. One unit was defined as the amount of enzyme catalyzing the incorporation of 1 μmole of glycine/mg of protein nitrogen of particles per hour at 30° over a blank without enzyme, and the specific activity expressed as units per mg. of protein. Protein was determined by the biuret method[19] or spectrophotometrically.[20]

For purification of the incorporation enzyme the combined extract (from 70 gm. of cells) obtained after removing the particles by centrifugation at top speed of the Servall angle centrifuge, as already described, was adjusted from a protein con-

centration of 33 mg/ml to 10 mg/ml by dilution with ice-cold 0.01 *M* phosphate buffer, pH 7.4, and chilled in ice. Solid ammonium sulfate was added with mechanical stirring at 0° to 0.25 saturation, and the precipitate was removed by centrifugation and discarded. The enzyme was precipitated by raising the ammonium sulfate concentration of the supernatant solution to 0.55 saturation. The precipitate was dissolved in ice-cold 0.01 *M* phosphate buffer, pH 7.4, and dialyzed overnight, at 4°, against 2 liters of the same buffer. The dialyzed solution, containing 26 mg. of protein/ml, was adjusted to 10 mg/ml with the above phosphate buffer and to pH 5.1 with 0.1 *M* acetate buffer, pH 4.2. This solution was stirred for 10 minutes at 0° with calcium phosphate gel (prepared by the Sigma Chemical Company, St. Louis, Missouri) in the proportion of 7 ml. of gel suspension (40 mg. of $Ca_3(PO_4)_2$ per ml.) per 25 ml. of enzyme solution. The gel was collected by centrifugation, eluted with 5 ml. of 0.1 *M* phosphate buffer, pH 6.0, for every 7 ml. of gel suspension used, and the eluate dialyzed for 2 hours at 4° against 500 ml. of 0.01 *M* phosphate buffer, pH 7.4. To 5-ml. aliquots of the dialyzed eluate (containing 9 mg. of protein/ml) was added, with stirring for 2 minutes at 0°, 0.7 ml. of 1 per cent protamine sulfate adjusted to pH 5.0. The precipitate was removed by centrifugation and discarded, and the supernatant solution dialyzed against phosphate buffer as above. The resulting solution was refractionated with ammonium sulfate and the fraction at 0.55 saturation discarded. The supernatant solution, containing the enzyme, was freed of ammonium sulfate by dialysis against 0.01 *M* phosphate buffer, pH 7.4, and stored at −18°. The results of a typical fractionation are summarized in Table 1.

TABLE 1

PURIFICATION OF AMINO ACID INCORPORATION ENZYME OF *Alcaligenes faecalis**

Step	Volume (Ml.)	Units†	Protein (Mg.)	Specific Activity‡	280§/260	Yield (Per Cent)
1. Extract	300	338	9900	0.03	0.58	100
2. 1st. $(NH_4)_2SO_4$ fractionation (0.25–0.55)	120	292	5520	0.05	0.60	86
3. $Ca_3(PO_4)_2$ gel eluate	24	236	255	0.92	0.63	70
4. Protamine supernatant	25	200	60	3.7	1.00	59
5. 2nd $(NH_2)SO_4$ fractionation (0.55 supernatant)	25	192	8	24.0	1.00	57

* 70 gm. of cells.

† Assayed with C^{14}-glycine. The assay of fractions at steps 1 through 4, with high nucleic acid content, refers to heat-labile activity; at steps 4 and 5, all the activity of the fractions is heat-labile.

‡ Units per mg. of protein.

§ Ratio of absorption of light at wave length 280 mμ to that at 260 mμ.

When assayed for incorporation of C^{14}-valine, the initial extract contained 220 units of specific activity 0.02 and the final fraction 41 units of specific activity 5.1. It is not possible to decide at this time whether the difference in yield of glycine and valine activity is due to partial separation of individual activities or to the low accuracy of the assay method particularly with crude enzyme fractions. The fractions of specific activity (glycine) 24 are free of polynucleotide phosphorylase and of enzymes catalyzing the amino acid–dependent exchange of PP^{32} with ATP, although these enzymes are quite active in the initial extract.[21] Table 2 illustrates the removal of amino acid activating enzymes on purification of the incorporation enzyme. Similar results have been obtained with each of the remaining amino

acids not shown in the table. The activating enzymes still present at step 4 (Table 2) are found in the 0–0.55 ammonium sulfate fraction discarded at step 5.

The activity of the incorporation enzyme is completely destroyed by heating for 3 minutes at 100°. Preparations up to step 3 keep their activity unchanged for several weeks when stored at −18°, but the purest preparations are exceedingly unstable. They lose about half of the activity on storage at −18° for 8–10 days and most of the activity after about 2 weeks, even if repeated freezing and thawing are avoided.

TABLE 2

REMOVAL OF AMINO ACID–ACTIVATING ENZYMES ON PURIFICATION OF INCORPORATION ENZYME*

	Purification Step†			
L-Amino Acid Substrate	1. Extract	3. Gel eluate	4. Protamine	5. 2d $(NH_4)_2SO_4$
Mixture of 19	36.4	34.2	17.0	0
Glycine	1.6	2.3	1.1	0
Threonine	2.7	0.1	0.1	0
Valine	14.4	18.2	15.0	0
Leucine	13.9	12.5	4.1	0
Lysine	5.6	0	0	0
Proline	15.4	0.5	0.5	0

* The assay samples for amino acid activation (exchange of P^{32}-labeled pyrophosphate with ATP in the presence of amino acid) contained per ml.: Tris buffer, pH 7.1, 100 μmoles; ATP, 3 μmoles; PP^{32}, 3 μmoles (60,000 to 200,000 cpm); $MgCl_2$, 10.0 μmoles; KF, 5 μmoles; L-amino acid, 2–3 μmoles; and enzyme fraction with 0.1 mg. of protein. Incubation, 20 minutes at 37°. Results expressed as per cent exchange over a blank without added amino acid. The blank varied from 0.6 to 0.8, with fractions from steps 1, 3, and 4, and was 0.3 with fraction from step 5. The blank without enzyme was 0.3 throughout.

† Cf. Table 1.

TABLE 3

EFFECT OF AMINO ACID–INCORPORATION ENZYME ON INCORPORATION OF C^{14}-LABELED AMINO ACIDS INTO PROTEIN OF *Alcaligenes* PARTICLES*

	Labeled Amino Acid Incorporated into Particles (Millimicromoles per Mg. Protein N of Particles per Hour)				
			Washed Particles		
Added Labeled Amino Acid	Unwashed Particles (*a*)	No Addition (*b*)	Amino Acids (*c*)	Enzyme (*d*)	Amino Acids + Enzyme (*e*)
Glycine	57	17	47	49	59
D,L-Alanine	40	16	...	30	...
L-Threonine	310	116	166	245	270
D,L-Valine	13	7	13	12	17
D,L-Leucine	24	12	19	20	22
L-Lysine	45	23	...	50	...
L-Proline	458	338	...	415	...
D,L-Phenylalanine	42	10	...	25	...
L-Tyrosine	40	18	...	36	...
D,L-Tryptophan	45	15	30	28	38

* Conditions as in Fig. 1 with 0.25–0.5 μmole (6,000–30,000 cpm) of labeled amino acid. Unwashed particles (*a*). Washed particles with no further additions (*b*). Washed particles with addition of either a mixture of non-labeled 18 L-amino acids (making a total of 19 with the labeled amino acid present in a given experiment), each 0.3 μg. (*c*), amino acid incorporation enzyme, specific activity (glycine) 24, with 10 μg. of protein (*d*), or both (*e*). The L-amino acids used were glycine, alanine, serine, threonine, valine, leucine, isoleucine, cysteine, cystine, methionine, arginine, lysine, proline, phenylalanine, tyrosine, tryptophan, histidine, aspartic acid, and glutamic acid.

Activity of Incorporation Enzyme.—The incorporation enzyme stimulates to a varying degree the incorporation of all the labeled amino acids, so far tested, into protein of washed *A. faecalis* particles. Typical results with highly purified enzyme are shown in Table 3. Similar stimulation of the incorporation of some labeled amino acids is produced by a mixture containing small amounts of the non-labeled remaining 18 amino acids. The stimulation due to the joint addition of amino acids and incorporation enzyme is somewhat higher than that produced by either alone, and, under these conditions, the incorporation of labeled amino acid

approaches that obtained with unsupplemented, unwashed particles (Table 3). The results suggest that washing with NaCl makes the particles partially deficient in both free amino acids and incorporation enzyme. It has already been mentioned that the original supernatant extract increases the incorporation of amino acids to levels higher than obtained with unwashed particles alone and that most of this effect is due to heat-stable compounds. This suggests that the extract contains factors (possibly RNA), other than free amino acids and incorporation enzyme, involved in amino acid incorporation by the particles.

Incubation of the washed particles with an amino acid mixture leads to a significant increase in protein, which, as shown in Table 4, is larger in the presence of added incorporation enzyme.

TABLE 4

INCREASE OF PROTEIN ON INCUBATION OF *Alcaligenes* PARTICLES WITH AMINO ACIDS

SYSTEM	PROTEIN (μG.) Initial	Final	Increase
Complete*	474	575	101
No incorporation enzyme	435	510	75
No amino acids	475	525	50
No incorporation enzyme, no amino acids	435	465	30

* The complete system contained Tris buffer, pH 7.0, 50 μmoles; $MgCl_2$, 10 μmoles; K_2HPO_4, 40 μmoles; mixture of 19 L-amino acids, each 26 μg.; washed particles with 430–440 μg. of protein; and amino acid incorporation enzyme, specific activity (glycine) 24, with 40 μg. of protein. Final volume, 1.0 ml. Incubation in air, 1 hour at 30°. The amino acid mixture contained the amino acids listed in the legend to Table 3.

It appeared possible that the incorporation enzyme might be involved in oxidative phosphorylation and thus indirectly stimulate protein synthesis in the particles. This was not unlikely in view of Pinchot's work[22] implicating a soluble enzyme (or enzymes) in the oxidative phosphorylation system of *A. faecalis*. This possibility was ruled out in joint experiments with Dr. Pinchot, showing that the purified incorporation enzyme could not substitute for his soluble, heat-labile component, nor did it have any effect on the phosphorylation coupled to the oxidation of reduced diphosphopyridine nucleotide by the washed *Alcaligenes* particles used in this work. It would, therefore, appear that the incorporation enzyme is more or less directly concerned with some phase of protein biosynthesis.

DISCUSSION

The washed *Alcaligenes* particles seem to be devoid of activating enzymes catalyzing the amino acid–dependent exchange of PP^{32} with ATP, even if further disintegrated by prolonged sonic oscillation. Suspensions of disintegrated particles (2 mg. of protein per ml. of reaction mixture), when assayed under the conditions of Table 2, gave a blank value, i.e., 0.3 per cent exchange, in the absence as well as in the presence of an amino acid mixture. Thus the system described in this paper is capable of active incorporation of amino acids into particle protein in the apparent absence of conventional amino acid–activating enzymes. However, the occurrence of some kind of amino acid activation is indicated by the inactivity of the system under conditions in which oxidative phosphorylation cannot take place.

Although it has as yet not been ascertained whether the incorporated amino acids are present in peptide linkage in the particle proteins, the stimulation of the

incorporation of some labeled amino acids by a mixture of non-labeled amino acids and the observed small but significant net increases of protein are suggestive of the occurrence of true protein synthesis. These suggestions are reinforced by the observation (*a*) that the incorporation of amino acids is not reversible, since a labeled amino acid (valine, leucine, glutamic acid, phenylalanine), once incorporated, is not displaced by reincubation of the particles with a forty fold excess of the same unlabeled amino acid under conditions of active incorporation, and (*b*) the incorporation is inhibited by chloramphenicol, which is generally considered to be an inhibitor of protein biosynthesis.

SUMMARY

Particulate preparations of *Alcaligenes faecalis*, consisting largely of cell membrane fragments, incorporate amino acids into their proteins. This incorporation, which appears to reflect protein biosynthesis, is driven by oxidative phosphorylation and is stimulated by an enzyme, present in the supernatant extract, which has been isolated in highly purified form. The purified enzyme is free of activating enzymes catalyzing the amino acid–dependent exchange of PP^{32} with ATP and the same seems to be true of the bacterial particles.

We are indebted to Mrs. Monique Beljanski for skilful technical assistance.

* Aided by grants from the National Institute of Arthritis and Metabolic Diseases (Grant A-1845) and the National Cancer Institute (Grant C-2784) of the National Institutes of Health, United States Public Health Service; the Rockefeller Foundation; the American Cancer Society (recommended by the Committee on Growth, National Research Council); and by a contract (Nonr-285(31), NR 120-490) between the Office of Naval Research and New York University College of Medicine.

† Chargé de Recherches, Centre National de la Recherche Scientifique, on leave of absence from the Service de Biochimie Cellulaire, Institut Pasteur, 28 rue du Docteur Roux, Paris XV, France.

[1] M. B. Hoagland, P. C. Zamecnik, and M. L. Stephenson, *Biochim. et Biophys. Acta*, **24**, 215, 1957.

[2] M. B. Hoagland, E. B. Keller, and P. C. Zamecnik, *J. Biol. Chem.*, **218**, 345, 1956.

[3] Abbreviations: ATP, adenosine triphosphate; RNA, ribonucleic acid; AMP, adenosine-5′-monophosphate; PP, pyrophosphate; Tris, tris(hydroxymethyl)aminomethane; cpm, counts per minute.

[4] F. Lipmann and others, Symposium on amino acid activation, these PROCEEDINGS, **44**, 67, 1958.

[5] R. S. Schweet, F. C. Bovard, E. Allen, and E. Glassman, these PROCEEDINGS, **44**, 173, 1958.

[6] S. B. Weiss, G. Acs, and F. Lipmann, these PROCEEDINGS, **44**, 189, 1958.

[7] P. Berg, *J. Biol. Chem.*, **222**, 1025, 1956.

[8] J. A. De Moss and G. D. Novelli, *Biochim. et Biophys. Acta*, **22**, 49, 1956.

[9] B. Nismann, F. H. Bergmann, and P. Berg, *Biochim. et Biophys. Acta*, **26**, 639, 1957.

[10] G. C. Webster, *Arch. Biochem. Biophys.*, **70**, 622, 1957.

[11] E. W. Davie, V. V. Koningsberger, and F. A. Lipmann, *Arch. Biochem. Biophys.*, **65**, 21, 1956.

[12] E. F. Gale and J. P. Folkes, *Biochem. J.*, **59**, 661, 675, 1955.

[13] M. Beljanski, *Biochim. et Biophys. Acta*, **15**, 425, 1954.

[14] S. Spiegelman, in *The Chemical Basis of Heredity* (Baltimore: Johns Hopkins Press, 1955), p. 232.

[15] G. E. Connell, P. Lengyel, and R. C. Warner, submitted to *Biochim. et Biophys. Acta*.

[16] G. B. Pinchot, *J. Biol. Chem.*, **205**, 65, 1953. We are indebted to Dr. Pinchot for a culture of the organism.

[17] V. G. Allfrey and A. E. Mirsky, these PROCEEDINGS, **43**, 589, 1957.

[18] S. Mii and S. Ochoa, *Biochim. et Biophys. Acta*, **26**, 445, 1957.
[19] A. G. Gornall, C. J. Bardawill, and M. M. David, *J. Biol. Chem.*, **177**, 751, 1949.
[20] O. Warburg and W. Christian, *Biochem. Z.*, **310**, 384, 1941.
[21] To a greater or lesser extent the extract promoted the PP^{32}-ATP exchange with each of the 19 amino acids tested.
[22] G. B. Pinchot, *J. Biol. Chem.*, **229**, 1, 1957.

---

*VITAMIN K, AN ESSENTIAL FACTOR FOR THE PHOTOCHEMICAL ACTIVITY OF ISOLATED CHLOROPLASTS**

BY NORMAN I. BISHOP

DEPARTMENT OF BIOCHEMISTRY (FELS FUND), UNIVERSITY OF CHICAGO

Communicated by James Franck, April 18, 1958

Introduction.—The site of photosynthesis in green plants is the chloroplast. Besides the indispensable chlorophyll *a*, the chloroplasts always contain one or more carotenoids. For a long time this fact has prompted questions concerning their function in the mechanism of photosynthesis, since some of these carotenoids are also known to contribute to effective light absorption.[1] Otherwise nothing definite is known about the essential catalytic role of the ubiquitous carotenoids. Griffiths *et al.*[2] demonstrated that the photoreduction of a carotene-free strain of *Rhodopseudomonas spheroides* proceeds normally under anaerobic conditions. Under aerobic conditions the deficient bacterial cells were photoxidized, while the normal strain survived and grew during a light period in air. A discussion has ensued, consequently, on whether this action—the prevention of photoxidation—is the only function of the carotenes.

The recent observations of French and Lynch[3] suggest that there may be another function for the yellow pigments. They showed that freeze-dried chloroplasts lose most of their ability to reduce a dyestuff in the light by the extraction of some constituents with cold petroleum ether. The photochemical activity could be restored by readdition either of the extract or of β-carotene preparations. The obvious conclusion seemed to be that β-carotene was an essential intermediate for the dye reduction and, implicitly, for the effective photolysis of water. However, the extract was much more efficient than β-carotene.

Since our interests have been focused on the mechanism of oxygen production,[4,5] we considered the experiments of French and Lynch important enough to be repeated carefully. Not only were they reproducible, but it became apparent that the noted results applied to the photolysis of water (Hill reaction) in general, since oxygen evolution is also reversibly dependent on the presence of petroleum ether–extractable substances. Careful purification of the petroleum ether extract of chloroplasts as well as of commercially available carotenes and subsequent testing of the photochemical activity of extracted material with potassium ferricyanide as the oxidant showed, however, that the carotenes themselves were not, or not entirely, responsible for the reactivation effect. We have, therefore, looked for other petroleum ether–soluble substances with likely catalytic properties and have found that certain vitamin K-type compounds possess such characteristics.

Materials and Methods.—The spinach chloroplast fragments used for our experi-

ments were prepared by the method of Spikes.[6] The sedimented chloroplasts in the final centrifugation were rinsed twice in glass-distilled water and then resuspended in this medium, frozen rapidly at −78° C. and lyophilized. The freeze-dried material was stored at −25° C.

The photochemical activity of the chloroplast fragments (lyophilized and extracted) was measured by a potentiometric technique previously described.[7] Illumination was provided by a 750-watt projection bulb which gave a maximum white-light intensity of 27,000 lux. The general reaction system (final volume: 12

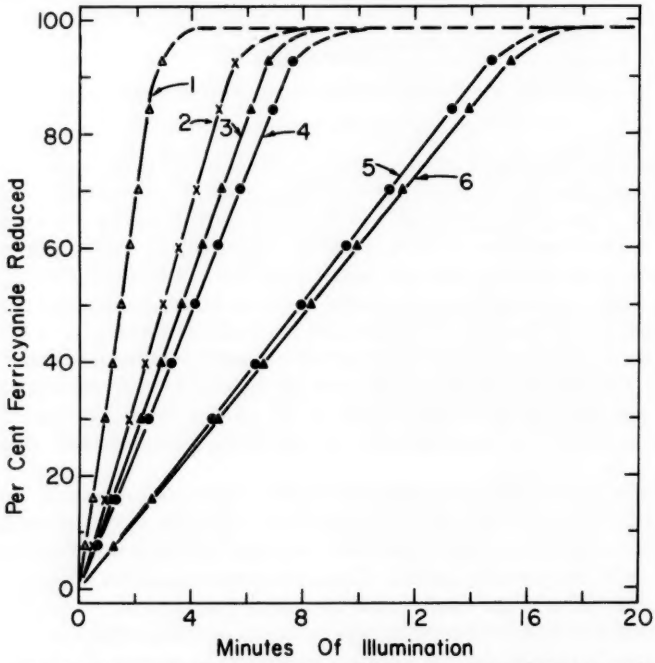


FIG. 1.—Curves showing the effect of addition of various substances on the photochemical activity of petroleum ether extracted chloroplasts. No. *1*, control, no extraction; No. *2*, 0.1 mg. Menadione; No. *3*, petroleum ether extract; No. *4*, "partially purified" β-carotene; No. *5*, 0.1 mg. purified β-carotene; No. *6*, extracted chloroplasts. Temperature, 15° C. Gas phase, prepurified nitrogen.

ml.) contained chloroplast fragments equivalent to 8×10^{-5} *M* chlorophyll and also 5×10^{-4} *M* potassium ferricyanide, 0.01 *M* potassium chloride, 0.17 *M* sucrose, 0.50 *M* potassium phosphate buffer at pH = 7.0.

Gas changes were measured by conventional manometric techniques. For the experiments reported in this paper, conical vessels fitted with double side arms and center well were used.

Samples of the freeze-dried chloroplasts were extracted with cold petroleum ether (4° C.) in a tube containing a fine fritted-glass filter that would not pass any of the chloroplasts. After extraction, the excess petroleum ether was evaporated under a stream of nitrogen.

The petroleum ether used throughout our experiments had first been washed with concentrated sulfuric acid, then several times with water, and then dried with sodium sulfate.

Fractionation of the petroleum ether extract of the dried chloroplasts was performed first on a powdered-sugar column. With petroleum ether containing 5 per cent benzene as the eluant, three yellow bands developed. The eluant was allowed to run through the column until the yellow bands had separated sufficiently to be collected individually. The first fraction to come through the column was a mixture of yellow pigments devoid of xanthophylls. This was considered as "partially purified" β-carotene. The carotene fraction was separated further on a magnesium oxide–Celite column using *n*-Hexane-1 per cent acetone as the eluant. The α- and β-carotene from carrots was prepared by the method outlined by Karrer and Jucker.[8]

The method for readdition of the various compounds was as follows: A weighed portion of the extracted chloroplasts was placed in a fritted-glass funnel (fine), and a gentle flow of prepurified nitrogen was directed from below. Aliquots (usually 5 ml.) containing the desired concentration of the substance being investigated were allowed to flow slowly onto the extracted material. The flow of nitrogen was adjusted so that all the petroleum ether was evaporated in approximately 15 minutes. The dried material was resuspended in 0.25 *M* sucrose, 0.015 *M* KCl, and 0.075 *M* phosphate buffer (pH = 7) and frozen rapidly in an acetone–dry-ice bath in order to have comparable material for several tests. Prior to a rate determination, potassium ferricyanide was added after the chloroplast suspension had been thawed.

Results.—One of the many petroleum ether–soluble compounds present in green cells is vitamin K. It is well known that the main source of this vitamin is green plant material. Furthermore, it is located in the chloroplasts and not in the cytoplasm.[9] Arnon *et al.*[10] found that it may serve as a catalyst or co-factor in photophosphorylation. Here its effect is rather unspecific, since other oxido-reduction catalysts can act as substitutes.[11] Because he found vitamin K in chloroplasts, Dam[9] suggested that it might be involved in photosynthesis. Wessels[12] proposed a mechanism whereby vitamin K could participate both in the Hill reaction and in photophosphorylation. Neither, however, offered any direct experimental proof of their hypotheses.

In our experiments we added several compounds having vitamin K activity to freeze-dried chloroplasts that had been extracted with petroleum ether at 4° C. The influence of one of the compounds, Menadione, is shown in Figure 1. Rate measurements were made with the technique described above. It is evident that Menadione (vitamin K_3) is capable of restoring the photochemical activity to extracted chloroplasts under conditions where the effect of purified α- and β-carotene is negligible by comparison. Partially purified β-carotene from the chloroplast extract, however, is as effective as the vitamin K_3. Similar results were obtained using vitamin K_5. A mixture of 0.1 mg. Menadione and 0.1 mg. β-carotene reactivated the chloroplast activity to about the same extent as did the vitamin K_3 alone. Repeated chromatographic purification of the extracted β-carotene fraction eventually yields a carotene which is ineffective in restoring photochemical activity. If the source of the carotenes is carrots rather than green plant material, then this careful purification is not required to show this; it is well known that carrots have a

low content of vitamin K.[9] These findings offer a convenient explanation for the variability of certain commercial preparations of β-carotene in restoring the photolytic capacity to chloroplasts devoid of their yellow pigments.[13]

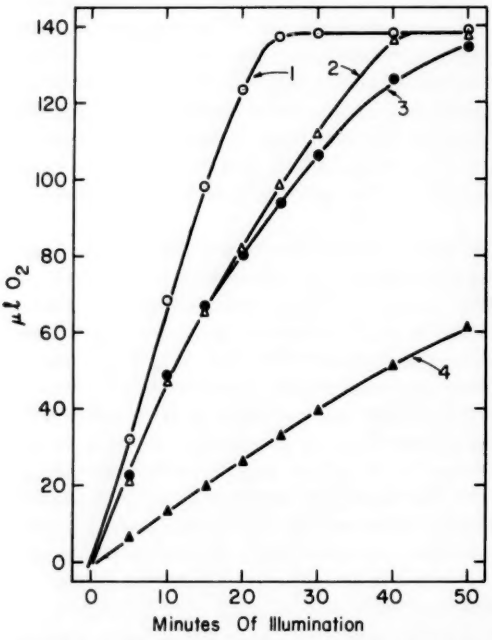


FIG. 2.—Curves illustrating the capacity of Menadione and of the petroleum ether extract to restore the oxygen-producing power of illuminated chloroplasts. No. *1*, control; No. *2*, petroleum ether extract; No. *3*, 1 mg. Menadione; No. 4, extracted chloroplasts. Temperature. 10° C. Gas phase, prepurified nitrogen.

Proof that the substituted naphthoquinones are not inducing an artificial photo-reduction of the added oxidant but are essential for the evolution of oxygen is shown in Figure 2. Menadione is as efficient as the chloroplast extract in restoring the photochemical activity in terms of oxygen production. This system, however, is quite sensitive to oxidation, and the initial rate of oxygen production falls off. Further experiments are required in order to determine whether vitamin K is participating directly in the oxygen pathway, that is, in the dismutation of "Y·OH," or whether it is acting as a hydrogen transfer agent for photosynthesis. Our experiments, on the one hand, make it unlikely that the carotenoids function in the particular way the various naphthoquinones do and, on the other, indicate a new and rather specific role for vitamin K in photosynthesis.

* The work described in this paper was supported by the United States Atomic Energy Commission under contract # AT (11-1)-239.

[1] Rabinowitch, E., *Photosynthesis and Related Processes*, Vols. 1 and 2, New York (1945).
[2] M. Griffiths, W. R. Sistrom, G. Cohen-Bazire, and R. Y. Stanier, *Nature*, **176,** 1211, 1955.
[3] V. H. Lynch and C. S. French, *Arch. Biochem. and Biophys.*, **70,** 382, 1957.
[4] N. I. Bishop, *Biochim. et Biophys. Acta*, **27,** 205, 1958.
[5] E. Kessler, *Arch. Biochem. and Biophys.*, **59,** 527, 1955.
[6] J. D. Spikes, *Arch. Biochem. and Biophys.*, **35,** 101, 1952.
[7] J. D. Spikes, R. Lumry, J. S. Rieske, and R. J. Marcus, *Plant Physiol.*, **29,** 161, 1954.
[8] P. Karrer and E. Jucker, *Carotenoids* (Amsterdam, 1950).
[9] H. Dam, *Advances in Enzymol.*, **2,** 285, 1942.
[10] D. I. Arnon, F. R. Whatley, and M. B. Allen, *J. Am. Chem. Soc.*, **76,** 6324, 1954.
[11] A. Jagendorf, *Personal Communication.*
[12] J. S. C. Wessels, *Rec. trav. chim.*, **73,** 529, 1954.
[13] M. Milner, *Carnegie Institute of Washington Year Book*, **56,** 271, 1957.

IONIZATION OF INDIVIDUAL GROUPS IN DIBASIC ACIDS, WITH APPLICATION TO THE AMINO AND HYDROXYL GROUPS OF TYROSINE

By John T. Edsall, R. Bruce Martin, and Barbara R. Hollingworth*

BIOLOGICAL LABORATORIES, HARVARD UNIVERSITY, CAMBRIDGE, MASSACHUSETTS

Read before the Academy, April 28, 1958

A dibasic acid (HAH) with two acidic groups ionizes according to the following scheme (Fig. 1):

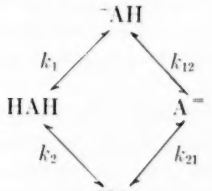


FIG. 1. Scheme for the ionization of a dibasic acid, showing the meaning of the four "microscopic" constants. The symbol H on the left of HAH is the acidic proton denoted as (1); that on the right, as (2).

The individual, or microscopic, ionization constants, such as k_1, are defined by the relations $k_1 = (H^+)\ (^-AH)/(HAH)$; $k_{12} = (H^+)\ (A^=)/(^-AH)$; etc. Here (H^+) denotes the hydrogen ion activity—that is, it is defined in terms of the measured pH by the equation $(H^+) = 10^{-pH}$—and the concentrations of the acid and its conjugate bases are denoted by the appropriate symbols in parentheses.[1] The minus signs simply denote that, if the net charge of HAH is Z proton units, then the net charge of ^-AH or HA^- is $Z - 1$, and that of $A^=$ is $Z - 2$. Thus for succinic acid $Z = 0$; for the glycine cation $Z = +1$; for the ethylenediammonium ion $Z = +2$. The notation used for the microscopic constants follows that of Hill;[2] the last numeral in the subscript denotes the group which donates a proton in the indicated process (in this case either 1 or 2), while the numerals which precede this indicate the groups, if any, from which protons have previously been removed.[3]

An ordinary titration, in which pH is determined as a function of the mean number of protons removed from HAH by addition of hydroxyl ion, does not give the microscopic constants directly but does yield two macroscopic constants, K_1 and K_2, which bear definite relations to the microscopic constants.[3, 4, 5, 6] They are defined by the relations:

$$K_1 = \frac{(H^+)\ [(^-AH) + (HA^-)]}{(HAH)} = k_1 + k_2, \tag{1}$$

$$K_2 = \frac{(H^+)\ (A^=)}{[(^-AH) + (HA^-)]}; \qquad K_2^{-1} = k_{12}^{-1} + k_{21}^{-1}. \tag{2}$$

It is apparent from Figure 1 and equations (1) and (2) that

$$k_1k_{12} = k_2k_{21} = K_1K_2 \equiv \frac{(H^+)^2(A^=)}{(HAH)}. \tag{3}$$

The course of the titration, expressed in terms of $\bar{h}$, the mean number of protons removed per molecule of dibasic acid, as a function of (H^+) and the K's, is

$$\bar{h} = \frac{\frac{K_1}{(H^+)} + \frac{2\,K_1K_2}{(H^+)^2}}{1 + \frac{K_1}{(H^+)} + \frac{K_1K_2}{(H^+)^2}}. \qquad (4)$$

The determination of K_1 and K_2 from the titration data is a well-known and straightforward procedure.[7] However, the knowledge of these constants and of the relations given in equations (1), (2), and (3) is insufficient to determine the four microscopic constants. One additional relation is required for the solution, and it is desirable to establish further relations, in order to obtain independent checks of the values for the microscopic constants. Two principal approaches to the problem have been employed.

First, one may convert one of the two acidic groups in HAH to an alkyl derivative RAH, or HAR′, where R and R′ generally denote methyl or ethyl groups. It has often been assumed that the effect of the group RA— on the ionization of hydrogen (2) is equivalent to that of HA—. If the left-hand hydrogen of HAH is the acidic hydrogen of a carboxyl group, and if RAH is the monoester of HAH, this is the well-known principle of Wegscheider, for which there is much supporting evidence.[4, 5, 8] When the assumption of this equivalence is justified, the pK value of HAR′ is equal to pk_1 in Figure 1, and that of RAH is equal to pk_2. This method of analysis has been used to determine the complete ionization scheme for simple amino acids with one amino and one carboxyl group,[4, 5] and a straightforward extension of the argument has been used to obtain a complete ionization scheme for the relations between all the eight microscopically different forms of glutamic acid.[2, 9]

An acidic group, although capable of releasing only one proton under the experimental conditions, may contain several potentially acidic hydrogens. The most familiar example is the $-NH_3^+$ group, which may be converted to $-NH_2$ by removing any one of the three hydrogens. In this case it is necessary to block all three potentially ionizable hydrogens, converting the $-NH_3^+$ to an $-NR_3^+$ group. This procedure has been employed by Grafius and Neilands,[10] who determined the pK value of the thiol group in N-trimethyl cysteine, in order to infer the ionization constant of the same group in cysteine, in the presence of a positively charged ammonium group. We have made similar use of N-trimethyl tyrosine, in a study mentioned below and to be reported later in detail, to determine the effect of a positively charged group on the ionization of the phenolic hydroxyl.

The assumption that the effect of RA— on the acidic groups is equivalent to that of —HA is open to criticism on at least two grounds. In the first place, HAR′ is more bulky than HAH. If the molecule is regarded as a cavity of low dielectric constant in the aqueous medium,[11] then the replacement of HA— by RA— increases the size of the cavity and should to some extent decrease the effective dielectric constant (D_E) for interaction between the groups. However,[11, 12] D_E is chiefly a function of the distance (d) between any one of the charged groups and the surface of the molecule. This distance appears to be generally close to 1.0 A and varies little from one molecule to another. Variation in the total molecular volume

has a relatively slight effect on D_R, if d is unaltered. In the second place, the HA— group may interact with the solvent by hydrogen bonding; the replacement of H by R inevitably eliminates some actual or potential hydrogen bonds and introduces other modifications in the configuration of the water molecules adjoining the alkyl group. Hydrogen bonding may also occur between two neighboring acidic groups if distances are suitable; after a proton has been removed from one of the groups, the second acidic proton may be held by hydrogen bonding between the groups. In such cases the esterification of one group may markedly decrease the ionization constant of its neighbor. This appears to be the case for maleic acid, for instance.[13]

The pK value for O-methylglycolic acid (3.53) is 0.3 pH units more acid than that of glycolic acid (3.83). This difference may be due to hydrogen bonding of water molecules around the hydroxyl group of glycolic acid. The pK value of S-methylthioglycolic acid (3.72) is, however, almost identical with the value (3.67) for the carboxyl group of thioglycolic acid;[13] this gives some direct justification for the equivalence postulate as applied to the —SH and —SCH_3 groups.

SPECTROPHOTOMETRIC DETERMINATION OF MICROSCOPIC CONSTANTS

Application to Tyrosine.—The second approach to the determination of the microscopic constants is by spectrophotometry. It requires that one kind of acidic group in the molecule shall differ greatly from its conjugate base in certain characteristic frequencies of its absorption spectrum, or of other types of spectra, such as Raman spectra,[14] or nuclear magnetic resonance spectra. Preferably a frequency is chosen for which one form (say the conjugate base) shows strong absorption or a strong Raman line, whereas the other shows no absorption or Raman line. It is, of course, essential that the other groups in the molecule give rise to no interfering absorption, or Raman line, in either the acid or the conjugate basic form. The aim of the procedure is to measure the total concentration of all forms in which the particular group in question is in either the acid or the basic form, as a function of pH. A notable example of this approach is given by the work of Benesch and Benesch[15] on the sulfhydryl group of cysteine. They made use of the ultraviolet absorption of the —S^- group in the region between 2300 and 2400 A, and used an algebraic method to calculate the microscopic constants from their data. Much earlier, Brand, Goldwater, and Zucker,[16] in a pioneer study, determined the microscopic constants of the amino and hydroxyl groups of tyrosine, making use of the well-known ultraviolet absorption of the ionized phenolic hydroxyl group with a maximum near 2950 A. Their data were, unfortunately, reported only in a brief preliminary abstract and have not attracted the attention they deserved. We have reinvestigated the problem, in order to determine the effects involved with more precision and with several independent checks, and also have formulated a method of analysis of the data which permits the evaluation of the microscopic constants with perhaps more simplicity than by other methods.

Tyrosine and cysteine are, of course, tribasic, not dibasic, acids. However, if we consider only the ionization which occurs at pH values above 6 or 7, it is safe to assume that the carboxyl group carries a negative charge, in all the microscopic forms under consideration. In this case we deal only with the ionization of two groups (—SH and —NH_3^+ in cysteine; —OH and —NH_3^+ in tyrosine) and may

regard these molecules in effect as dibasic acids. The course of ionization of the two groups can then be described for the specific case of tyrosine by a scheme equivalent to that given in Figure 1 and illustrated in Figure 2 below.

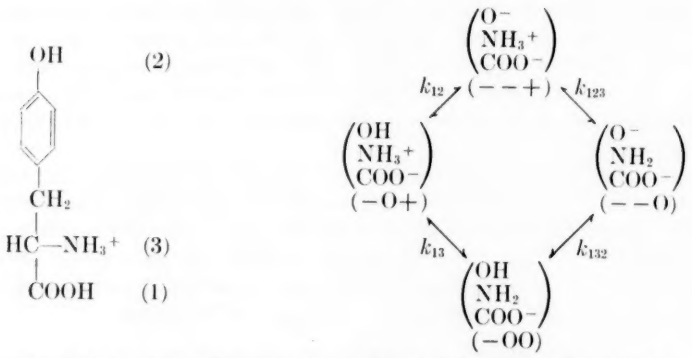


FIG. 2. Ionization of the ammonium and hydroxyl groups of tyrosine, the carboxyl group being already ionized. The numbering of the acidic groups is shown in the formula at the left.

We denote the three acidic groups in the molecule—carboxyl, hydroxyl and ammonium—as 1, 2, and 3, respectively, and denote the ionization constants by the notation of Hill,[2] already described. Since we consider only forms in which the carboxyl group is ionized, the subscript "1" appears in all microscopic constants indicated in Figure 2. Then the ionization scheme for the —OH and $—NH_3^+$ groups may be written (Fig. 2) thus:

These microscopic constants are related to the macroscopic constants generally denoted by K_2 and K_3, and equations (1), (2), and (3) become:

$$K_2 = k_{12} + k_{13}, \tag{5}$$

$$K_3^{-1} = k_{123}^{-1} + k_{132}^{-1}, \tag{6}$$

$$k_{12}k_{123} = k_{13}k_{132} = K_2K_3 = \frac{(H^+)^2(---O)}{(-O^+)}. \tag{7}$$

The abbreviated symbols in parentheses (see Fig. 2) for the different microscopic forms such as $(--+)$ denote the state of charge on groups 1, 2, and 3, respectively, reading from left to right.

On ionization of the hydroxyl group the maximum in the absorption spectrum shifts from 2750 to 2930 A. The molar extinction coefficient at 2950 and 3000 A is practically zero for the forms in which the OH group is un-ionized, whereas it is large for the forms in which —OH has been converted to $—O^-$. We therefore define a function α_{OH}, which represents the fraction of all the phenolic hydroxyl groups in the solution which are ionized. This may be expressed as follows:

$$\alpha_{OH} = \frac{\text{(concentration of all molecules with OH ionized)}}{\text{(concentration of all molecular species)}}$$

$$= \frac{(---^{+}) + (---O)}{(--O^{+}) + (--OO) + (---^{+}) + (---O)}$$

$$= \frac{k_{12}/(H^{+}) + k_{12}k_{123}/(H^{+})^2}{1 + (k_{12} + k_{13})/(H^{+}) + k_{12}k_{123}/(H^{+})^2} = \frac{k_{12}/(H^{+}) + K_2K_3/(H^{+})^2}{1 + K_2/(H^{+}) + K_2K_3/(H^{+})^2}. \quad (8)$$

From this we readily obtain a function M_{OH}, which is defined as

$$M_{OH} \equiv \frac{(H^{+})\alpha_{OH}}{1 - \alpha_{OH}} = \frac{k_{12}(H^{+}) + k_{12}k_{123}}{(H^{+}) + k_{13}} = \frac{k_{12}(H^{+}) + k_{13}k_{132}}{(H^{+}) + k_{13}} \quad (9)$$

or, in logarithmic form,

$$pM_{OH} = pH - \log \frac{\alpha_{OH}}{1 - \alpha_{OH}} = -\log \frac{k_{12}(H^{+}) + k_{13}k_{132}}{(H^{+}) + k_{13}}. \quad (9.1)$$

The form of the function M_{OH} is completely determined by the ratios k_{12}/k_{132} and k_{13}/k_{12}. It is apparent from (9.1) that a plot of pM_{OH} against α_{OH}, or against pH, should extrapolate

in the limit $\alpha_{OH} = 0$ . . . or
$H^{+}) \rightarrow \infty$ . . . to pk_{12}, and
in the limit $\alpha_{OH} = 1$ . . . or
$(H^{+}) \rightarrow 0$ . . . to pk_{132}.

The character of the function defined by equation (9) or (9.1) is illustrated in detail by the curves shown in Figures 3 and 4. These curves are calculated taking $pk_{132} - pk_{12} = 1$; thus the intercepts at $\alpha = 0$ and $\alpha = 1$ coincide in all the curves of Figure 3. The different curves correspond to different values of the ratio k_{13}/k_{12}. The same family of curves for pM_{OH} is plotted against pH in Figure 4, the zero point on the abscissa scale being taken where pH = pk_{12}. Similar families of curves are obtained for other values of $pk_{132} - pk_{12}$. It is apparent from Figure 3 that, if $k_{13} \gg k_{12}$, the value of pM_{OH} rapidly rises to a value close to pk_{132} as α_{OH} increases. In this case it is easy to determine pk_{132} by extrapolation, but the extrapolation to pk_{12} is very uncertain. Conversely, if $k_{13} \ll k_{12}$, the values of pM_{OH} remain close to pk_{12} until α_{OH} approaches unity, when the curve shoots suddenly upward. Hence in this case pk_{12} can be determined accurately, but the extrapolation to pk_{132} is very uncertain. Both these constants can be determined with reasonable accuracy by this type of plot, however, if k_{12} and k_{13} are of the same order of magnitude; preferably, the ratio k_{13}/k_{12} should lie between 5 and $^1/_5$. Fortunately this condition is fulfilled both for tyrosine and for cysteine.

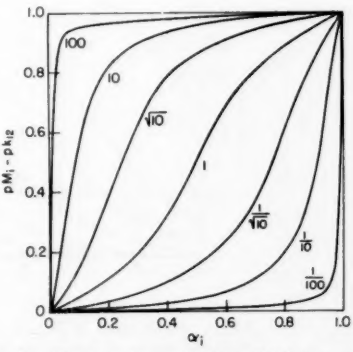


FIG. 3.—The function pM_i (eq. [9.1]) for a group denoted by i is represented as a function of the fractional ionization of that group, α_i. The zero of the ordinate scale is taken at $pM_i = pk_{12}$. All the curves shown correspond to an assumed value of $pk_{132} - pk_{12} = 1$ pH unit. The number attached to each curve represents the corresponding value of the ratio k_{13}/k_{12}.

Once k_{12} and k_{132} have been determined by the extrapolation procedure described above, it is then possible to take these two constants as known quantities and to solve for k_{13}. This may be done in various ways. For example, having drawn the curve which gives the best fit to the experimental data in a plot of pM_{OH} as a function of α_{OH}, we then read off from the curve the value of pM_{OH} corresponding to $\alpha_{OH} = 0.50$. At this point pM_{OH} = pH. Solving equation (9) for k_{13} under these conditions gives

$$k_{13} = \frac{(H^+)\ [k_{12} - (H^+)]}{(H^+) - k_{132}}, \text{ at } \alpha_{OH} = 0.5. \tag{10}$$

If $k_{13} \gg k_{12}$ or $k_{12} \gg k_{13}$, equation (10) may involve a small difference between two large numbers in the numerator or denominator. In this case we may proceed by

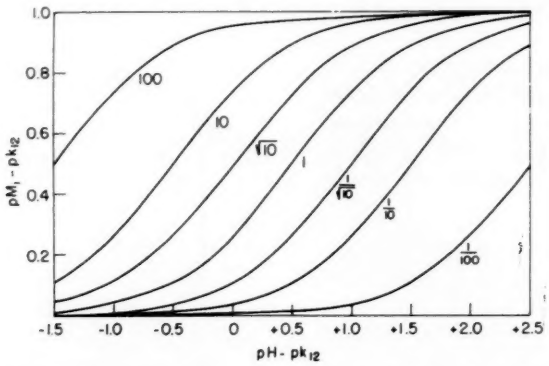


FIG. 4.—The data of Fig. 3, plotted as a function of pH. The ordinate scale is taken as in Fig. 3; the zero of the abscissa scale is taken at pH = pk_{12}.

considering the value of α_{OH} corresponding to the point at which $pM = (pk_{12} + pk_{132})/2$. Then for this particular value of $M = (k_{12}k_{132})^{1/2}$ we obtain from (9) and (9.1) the relation:

$$pk_{13} = \log \frac{\alpha_{OH}}{1 - \alpha_{OH}} + pk_{12}. \tag{11}$$

Since pk_{12} is already known, the determination of α_{OH} for this particular value of pM fixes pk_{13}.

Once a preliminary value of k_{13} has been derived by this method, a curve of the type shown in Figure 3 or Figure 4 may be calculated from the values of k_{12}, k_{13}, and k_{132}. A further slight adjustment of the estimated values of the constants may be required to give the best fit between the experimental data and the calculated curve. By this procedure and with the aid of equation (7), all four of the microscopic constants shown in Figure 2 can be derived, if α_{OH} is known as a function of pH over a sufficient range of experimental points.

If groups 2 and 3 were so far apart that they ionized completely independently of one another, we should have $pk_{12} = pk_{132}$ and $pk_{13} = pk_{123}$. In this case it is readily seen from equation (9) that M_{OH} would be a true constant, independent of

pH, and equal to k_{12} or k_{132}. This result holds regardless of the value of the ratio k_{13}/k_{12}.

In the calculation of α_{OH} it has generally been assumed that the molar extinction coefficient (which we denote by $\epsilon_{max.}$) of the form (— —O) is identical with that of the form (— —$^+$); in other words that the loss of a proton from the amino group does not affect the absorption spectrum of the ionized phenolic group. The value of $\epsilon_{max.}$ for (— —O) can be determined experimentally from light-absorption measurements, in very alkaline solutions, where pH is much larger than any of the *pk* values, but ϵ for (— —$^+$) is not directly measurable. There is good reason to doubt whether the assumption that ϵ(— —$^+$) = ϵ(— —O) can be exactly true. However we have tentatively adopted this simplifying assumption as a basis for calculation.

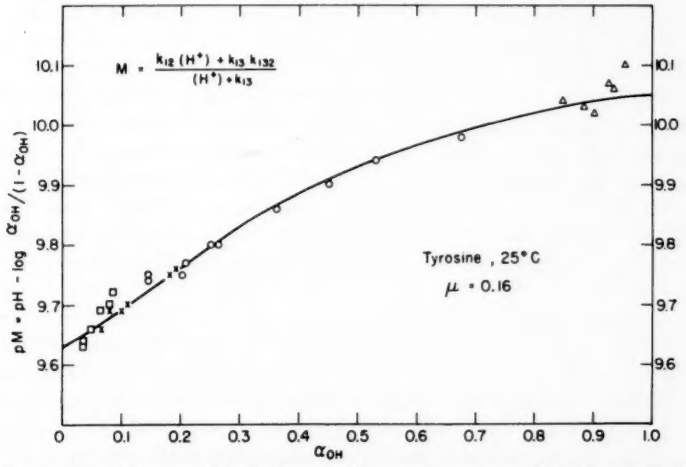


FIG. 5.—The ionization of the hydroxyl and ammonium groups of tyrosine, plotted by the method of Fig. 3. The buffers employed in the measurements are denoted as follows: ⊡, tris(hydroxymethyl) aminomethane; ○, glycine; x, ammonia; Δ, ε-aminocaproic acid. The pH range covered was from 8.2 to 11.4.

We also assume that the molar extinction coefficients (which we denote by ϵ_o) for the forms (—O$^+$) or (—OO) are equal.[17] Then, if ϵ is the observed extinction coefficient per mole of tyrosine present in all forms, we may write:

$$\alpha_{OH} = \frac{\epsilon - \epsilon_o}{\epsilon_{max} - \epsilon_o}. \tag{12}$$

Experimental Data on Tyrosine.—The results of our experimental determinations of the function *pM* for tyrosine at 25° and 0.16 ionic strength are shown in Figure 5. The tyrosine preparation used was obtained from the Mann Research Laboratories and was recrystallized twice from water before being used for these studies. The molar concentration of tyrosine was 0.001 *M* in most of the measurements, but the values obtained were independent of tyrosine concentration over more than a twofold range. The ionic strength was maintained constant by adding potassium chloride to the buffer solutions used to control the pH, so that the total ionic

strength was always 0.16. The buffers used are indicated in the legend to Figure 5; the total molar concentration of buffer acid plus conjugate base was generally 0.02 M. The pH measurements were made on a Beckman Model G pH meter and were calibrated with buffers recommended by the National Bureau of Standards.[18]

The function pM (eq. [9.1]) is obviously very sensitive to small errors in α_{OH} when α_{OH} is very close to zero or unity. Hence the points near the ends of the curve in Figure 5 show more scatter than those near the middle. However, extrapolations to $\alpha_{OH} = 0$ or 1 could be carried out with considerable confidence. By these extrapolations the value of pk_{12} was determined as 9.63 ± 0.02, and that of pk_{132} as 10.05 ± 0.03. Thus the placing of a positive charge on the amino group of tyrosine displaces the pK value of the hydroxyl group downward by 0.42. The curve drawn through the experimental points is calculated from these values of pk_{12} and pk_{132}, assuming $pk_{13} = 9.30$. Curves were also constructed for $pk_{13} = 9.25$ and 9.35, with unchanged values for pk_{12} and pk_{132}. The former lay above most of the experimental points, the latter mostly below them. The figure $pk_{13} = 9.30$ therefore appears to lie within ± 0.05 of the correct value. A slightly better fit to the experimental data would be obtained by assuming a slightly lower value, approximately 9.28, and we shall take this value for pk_{13} in subsequent calculations. We note that pk_{13} represents the pk of the ammonium group of tyrosine when the carboxyl group is ionized and the hydroxyl group un-ionized. If the hydroxyl group is also ionized, the corresponding pk value of the ammonium group is pk_{123}. From equation (7), using the values of the other constants given above, we find $pk_{123} = 9.70$.

The calculated values of α_{OH} and hence of the function M were derived by equation (14) from measurements of light absorption at both 2950 and 3000 A. The values of α_{OH} obtained for a given solution at these two wave lengths appear to be almost identical, although the values of pM obtained at 3000 A may, perhaps be slightly higher, by approximately 0.01 pH unit. In any case the discrepancy is small, and the results appear to justify the assumption that the forms ($——^+$) and ($——O$) have nearly the same extinction coefficient at these two wave lengths. The ratio $k_{13}/k_{12} = (—OO)/(——^+)$ is 2.3, from the values given above. Thus the form with the un-ionized hydroxyl group and a net charge of -1 predominates slightly over its isomer with one positive and two negative charges. The ratio of these two forms is, of course, independent of pH.

It is, of course, desirable to obtain further independent checks on the values of the microscopic constants derived by the analysis given above. Two additional experimental approaches were employed in checking these values.

1. A pH titration of isoelectric tyrosine with added alkali was carried out. This tiration yielded the two macroscopic constants K_2 and K_3. The results are as follows at an ionic strength of 0.16: $pK_2 = 9.11 \pm 0.01$, $pK_3 = 10.13 \pm 0.01$. By equations (5) and (6) these constants can also be calculated from the estimated values of the microscopic constants given above. The value for pK_2 calculated by equation (5) from pk_{12} and pk_{13} is 9.12, in excellent agreement with the value obtained directly from pH titration. The value for pK_3 calculated by equation (6) from pk_{123} and pk_{132} is 10.20. Here there is a significant, though not large, discrepancy between the macroscopic constant as determined directly and that determined from the microscopic constants and equation (6). As yet we can offer no

explanation for this discrepancy; corresponding titrations at a lower ionic strength (0.04) gave a very close agreement between pK_3 as determined directly and as inferred from the microscopic constant.

2. The equivalence postulate was employed, assuming tentatively that pK_2 for O-methyl tyrosine should be very nearly equal to pk_{13}, and that pK_2 for N-trimethyl tyrosine should be nearly equal to pk_{12}.[19] The values obtained at 0.16 ionic strength were:

O-methyl tyrosine $pK_2 = 9.27$ ($-NH_3^+$ group),
N-trimethyl tyrosine $pK_2 = 9.75$ ($-OH$ group).

The first of these values agrees extremely well with the pk_{13} as determined for tyrosine from the data shown in Figure 5. However, the value for N-trimethyl tyrosine is 0.12 pH units higher than pk_{12} as determined for tyrosine; the difference is well beyond experimental error. This difference is in the opposite direction to what might have been expected from the increased volume of N-trimethyl tyrosine as compared with tyrosine. This volume increase should tend to decrease the value of D_E in the Kirkwood-Westheimer theory.[11] Thus it appears that N-trimethyl derivative is not a particularly good model for tyrosine, in the forms containing a positively charged ammonium group. We may note that the pK value of the —SH group of N-trimethyl cysteine, as determined by Grafius and Neilands,[10] was found to be about 0.16 pH unit higher than the corresponding microscopic pk value for cysteine itself, as determined spectrophotometrically by Benesch and Benesch,[15] due allowance being made for the differences in ionic strength in the two studies.

On the other hand, O-methyl tyrosine appears to be an excellent model for the ionization of the amino group in tyrosine; the —OH and the $-OCH_3$ groups appear nearly equivalent in their effects on the $-NH_3^+$ group. As noted earlier in this paper, this equivalence does not hold for glycolic acid ($HO{\cdot}CH_2{\cdot}COOH$) and its O-methyl derivative; the latter has a pK value of 0.3 pH units *lower* than the unsubstituted acid. In tyrosine, the hydroxyl group is located much farther away from the other ionizing groups than in glycolic acid; moreover, it is an aromatic, not an aliphatic, hydroxyl group.

In general, our values for the microscopic constants agree reasonably well with those of Brand *et al.*:[16] a more detailed comparison will be given later, in a paper dealing with the esters and amides of tyrosine and its derivatives and with the effect of ionic strength on the various macroscopic and microscopic constants. Our values for the macroscopic constants agree well with those of Albert,[20] when due account is taken of the fact that his measurements were made at a much lower ionic strength than ours.

Many authors have assumed that the macroscopic pK_2 value of tyrosine represented the ionization of the ammonium group, and pK_3 the ionization of the hydroxyl group. In a rough approximate sense, this is nearer the truth than the reverse assignment would be. However, it is clear that a precise formulation of the true relations involved can be given only in terms of the microscopic constants.

In addition to the work of Brand *et al.*,[16] other studies have given indications of overlapping and interaction in the ionization of the amino and hydroxyl groups in tyrosine. For instance, Tanford and Roberts[21] determined the quantity we have

denoted by pM_{OH}, for $\alpha_{OH} = 0.25$, 0.50, and 0.75, at three different temperatures. Their data show clearly the increase of pM_{OH} with increasing α_{OH}; their values at 25° lie somewhat above our own data shown in Figure 5 and run more or less parallel to them. Possibly the difference between their data and ours is due to a difference in calibration of the pH measurements. In a recent personal communication, Professor Milton Levy reports independent observations closely similar to our own.

ELECTROSTATIC INTERACTIONS AND THE DISTANCE BETWEEN THE IONIZING GROUPS

The distance between the amino nitrogen and the hydroxyl hydrogen of tyrosine was found, by measurements on an accurate space model of the Pauling-Corey design, to lie between 6 and 8.5, with a best value near 7 A, according to the orientation of the hydroxyl group and of the bonds between the benzene ring and the amino group. The displacement of the pk value of the hydroxyl group by the positive charge on the amino group,[22] i.e., the value of $pk_{12} - pk_{132}$, should give a basis for calculating this distance, assuming one of the models proposed by Kirkwood and Westheimer.[11] Since tyrosine is clearly far from spherical, we have made use of their ellipsoidal model, assuming the two ionizing groups to be at the foci of the ellipsoid. Taking the molar volume of tyrosine to be 123.6 cc. (molecular volume 205 A^3),[23] we calculate the effective dielectric constant, D_E, for the interaction between the groups, to be 58. The Kirkwood-Westheimer equation is:

$$r = \epsilon^2/2.303\, D_E kT(\Delta\text{pK}). \qquad (13)$$

Here r is the distance between the two groups at the foci, ϵ is the proton charge, k is Boltzmann's constant, and T is the absolute temperature. The value of $\Delta\text{pK} = pk_{132} - pk_{12}$ is, as we have seen, 0.42 at ionic strength 0.16. The extrapolated value at zero ionic strength is, however, somewhat greater, on account of the difference in net charge between the various ionic species involved in the equilibria defined by these two pK values. We estimate ΔpK at zero ionic strength as 0.60. This value, substituted into equation (13), with $D_E = 58$, gives $r = 6.95$ A, a value which lies well within the upper and lower limits determined on the space model.

IONIZATION OF CYSTEINE

The scheme shown in Figure 2 applies directly to the ionization of cysteine and other sulfhydryl compounds, if we replace the hydroxyl by the sulfhydryl group, which we again denote as group 2. Benesch and Benesch[15] have determined α_{SH} for cysteine, cysteine ethyl ester, and cysteinyl glycine, as a function of pH, by light-absorption measurements between 2300 and 2400 A, and have calculated the four microscopic constants for each of these compounds by an algebraic method. They have kindly made their original data available to us, and we have calculated the function pM_{SH} (eq. [9.1]) as a function of α_{SH}. The results are shown in Figure 6; the curves are drawn using the microscopic pK values determined by Benesch and Benesch and are seen to give a very satisfactory fit to the experimental data. The striking difference between the curve for cysteine and that for its ethyl ester is immediately apparent; for the former the ratio k_{13}/k_{12} is nearly 0.5, for the latter it is 4.8; the resulting difference in the shape of the curves is as predicted; compare the illustrative set of curves in Figure 3.

The interaction between the sulfhydryl and ammonium groups in these three

cysteine derivatives is much greater than that between the hydroxyl and ammonium groups of tyrosine; $pk_{132} - pk_{12}$ lies between 1.50 and 1.65 in all three cases, a pk displacement about three times as great as in tyrosine. Measurement on a space model indicates the distance from the sulfhydryl hydrogen to the amino nitrogen to be less than 5 A, and probably considerably less, depending upon the orientation of the valence bonds.

We note that the titrations of Benesch and Benesch were not carried out at a fixed ionic strength; the ionic strength increased somewhat as the solution became more alkaline. Moreover, although they used an equation of the same form as (12)—with $\epsilon_o = 0$—to calculate α_{SH}, their ϵ values were not all determined at a single wave length but were taken in each case at the wave length of maximum absorption for the solution being measured. This wave length varied from near 2310 A at very low α_{SH} values to about 2370 A for $\alpha_{SH} = 1$. In this case it seems probable that the assumption that $\epsilon(- \; -O) = \epsilon(- \; -^{+})$ is not valid. The procedure adopted by Benesch and Benesch may correspond to the most reasonable simple assumption that can be made under the circumstances. The consistency of their results indicates that their values for the microscopic constants of sulfhydryl compounds should be nearly correct.

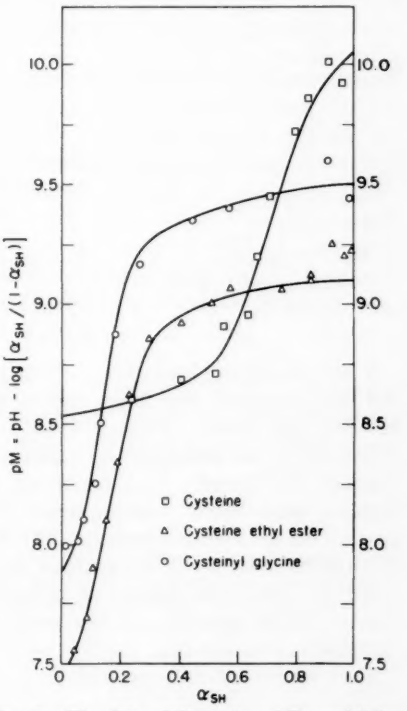


FIG. 6.—The data of Benesch and Benesch[15] for pM_{SH} in cysteine, cysteine ethyl ester, and cysteinyl glycine, plotted against α_{SH} by the method of equation (9.1) and Fig. 3; temperature 23°. The values for the microscopic constants given by Benesch and Benesch are as follows:

	pk_{12}	pk_{12}	pk_{132}	pk_{132}
Cysteine	8.53	8.86	10.36	10.03
Cysteine ethyl ester	7.45	6.77	8.41	9.09
Cysteinylglycine	7.87	7.14	8.75	9.48

Total Degree of Ionization of an n-Valent Acid.—A formula, somewhat analogous to equation (9), may be given for the macroscopic constants, K_1 and K_2, of a divalent acid, as defined by equations (1), (2), and (4). This is merely a special case of a general formula for the ionization of an n-valent acid H_nA, and we shall begin by deriving the general equation. In solution at a given pH, the various individual species derived from H_nA by removal of protons may be divided into classes, each being characterized by a number, h, which denotes the number of protons which must be removed from H_nA to give a molecule (or ion) of the class in question. Obviously, h may assume any value from zero (for H_nA) to n (for A^{n-}, with all protons removed).

A macroscopic ionization constant, K_t, is denoted by

$$K_i = (H^+)\ (\sum A^{i-})/(\sum A^{(i-1)-}). \tag{14}$$

Here $(\sum A^{i-})$ denotes the total concentration of all species from which i of the n acidic protons have been removed, and $(\sum A^{(i-1)-})$ denotes the total concentration of all species from which $i - 1$ of the n protons have been removed.[3]

In terms of these macroscopic constants, the mean number $(\bar{h})$ of protons removed, per mole of H_nA originally present, is:

$$\bar{h} = \frac{K_1/(H^+) + 2K_1K_2/(H^+)^2 + \ldots + (nK_1K_2 \ldots K_n)/(H^+)^n}{1 + K_1/(H^+) + K_1K_2/(H^+)^2 + \ldots + (K_1K_2 \ldots K_n)/(H^+)^n}. \tag{15}$$

We define α_{total}, the fraction of all acidic protons removed, as:

$$\alpha_{total} = \bar{h}/n. \tag{16}$$

In terms of α_{total}, we define a function M_{total}:

$$M_{total} = \frac{(H^+)\alpha_{total}}{1 - \alpha_{total}} = \frac{K_1 + 2K_1K_2/(H^+) + \ldots + (nK_1K_2 \ldots K_n)/(H^+)^{n-1}}{n + (n-1)K_1/(H^+) + (n-2)K_1K_2/(H^+)^2 + \ldots + (K_1K_2 \ldots K_{n-1})/(H^+)^{n-1}} \tag{17}$$

A plot of pM_{total} against α_{total} extrapolates to K_1/n as α_{total} approaches zero and (H^+) becomes very large, and to nK_n as α_{total} approaches unity and (H^+) becomes very small. This equation is actually equivalent to one derived by Scatchard for the association of a ligand with a molecule containing n sites which can bind that ligand. Scatchard's equation has been employed in earlier studies from this laboratory[24, 25] on the association of metallic ions with imidazoles, to determine the association constants. In those studies the equation was formulated in terms of association, rather than dissociation, constants and in terms of the quantity $\bar{\nu}$, the mean number of moles of ligand bound, per mole of binding substance. In the present case, the ligand is H^+ ion, $\bar{\nu} = n - h$, and $\bar{\nu}/(n - \bar{\nu})$ is equal to $(1 - \alpha_{total})/\alpha_{total}$.

For a dibasic acid (17) becomes:

$$M_{total} = \frac{(H^+)\alpha_{total}}{1 - \alpha_{total}} = \frac{K_1 + 2K_1K_2/(H^+)}{2 + K_1/(H^+)} = \frac{K_1(H^+) + 2K_1K_2}{2(H^+) + K_1}. \tag{18}$$

Thus a plot of pM_{total} against α_{total} should extrapolate to a limiting value of $K_1/2$ as α_{total} approaches zero, and to a limiting value of $2K_2$ as α_{total} approaches 1. The midpoint of the curve ($\alpha_{total} = 0.5$) is always at $pM_{total} = (pK_1 + pK_2)/2$, and the *form* of the curve, as distinct from its position on the pM scale, is determined only by the ratio K_1/K_2. If $K_1 = 4K_2$, representing the case in which the two ionizing groups are equivalent and independent, pM_{total} is a constant, equal to $K_1/2$ or $2K_2$, for all values of α_{total}; i.e., in this case pM_{total} is equal to the intrinsic pK of an individual group. If $K_1 \gg K_2$, the curve consists essentially of two horizontal straight lines, one corresponding to $pM_{total} = pK_1$, and the other to $pM_{total} - pK_2$, with a steep, nearly vertical, line connecting them at $\alpha_{total} = 0.5$. In such cases this method of analysis has relatively little use; it is likely to be most useful in practice,

as a method of evaluating K_1 and K_2, when the ratio K_1/K_2 lies between 4 and 40, or thereabouts.

SUMMARY

A method is formulated for calculating the four "microscopic" ionization constants required to describe the ionization of the individual groups in a dibasic acid, from spectroscopic data which measure the fractional ionization of one of the groups as a function of pH. This method is applied to the ionization of the amino and hydroxyl groups of tyrosine. It is found that placing a positive charge on the amino group decreases the pK value of the hydroxyl group by 0.42 pH units at ionic strength 0.16, and probably by about 0.60 pH units at zero ionic strength. There is, of course, a corresponding reciprocal effect of the ionization of the hydroxyl group on that of the ammonium group. These interaction effects are compatible with a Kirkwood-Westheimer ellipsoidal model, with a distance of 7 A between the amino and hydroxyl groups—a value which is physically reasonable.

Other methods of evaluating the microscopic constants, by alkyl substitution of some of the ionizable protons, are considered and applied to tyrosine derivatives. A brief discussion of the data of Benesch and Benesch, on the microscopic constants for the amino and sulfhydryl groups of cysteine, is also given.

We are indebted to Professor H. T. Clarke for providing us with a pure sample of O-methyl tyrosine and to Drs. Reinhold and Ruth Benesch for making available to us their original data on the ionization of the sulfhydryl group in cysteine and its derivatives.

* This work was supported by a grant from the National Science Foundation (G-3230) and from a United States Public Health Service grant (H-3169).

[1] The quantities denoted here by the k values are thus "apparent dissociation constants," which are often denoted by the symbol k', with appropriate subscripts; they are not the thermodynamic constants, which are expressed in terms of the activities of all components.

[2] T. L. Hill, *J. Phys. Chem.*, **48**, 101, 1944.

[3] For a more general discussion see J. T. Edsall and J. Wyman, *Biophysical Chemistry* (New York: Academic Press Inc., 1958, Vol. 1, chap. 9.

[4] L. Ebert, *Z. Physik. Chem.*, **121**, 385, 1926.

[5] J. T. Edsall and M. H. Blanchard, *J. Am. Chem. Soc.*, **55**, 2337, 1933.

[6] E. J. Cohn and J. T. Edsall, *Proteins, Amino Acids and Peptides* (New York: Reinhold Pub. Corp., 1943), chap. 4.

[7] See, for instance, H. M. Peek and T. L. Hill, *J. Am. Chem. Soc.*, **73**, 5304, 1951, and some further discussion later in the present paper.

[8] R. Wegscheider, *Monatshefte f. Chem.*, **16**, 153, 1895; **23**, 287, 1902.

[9] A. Neuberger, *Biochem. J.*, **30**, 2085, 1936.

[10] M. A. Grafius and J. B. Neilands, *J. Am. Chem. Soc.*, **77**, 3389, 1955.

[11] J. G. Kirkwood and F. H. Westheimer, *J. Chem. Phys.*, **6**, 506, 513, 1938.

[12] C. Tanford, *J. Am. Chem. Soc.*, **79**, 5348, 1957.

[13] H. C. Brown, D. H. McDaniel, and O. Häfliger, in *Determination of Organic Structures by Physical Methods*, ed. E. A. Braude and F. C. Nachod (New York: Academic Press, 1955), pp. 620, 632–634.

[14] See, for instance, J. T. Edsall, *J. Chem. Phys.*, **4**, 1, 1936; **5**, 225, 1937; J. T. Edsall and H. Scheinberg, *ibid.*, **8**, 520, 1940.

[15] R. E. Benesch and R. Benesch, *J. Am. Chem. Soc.*, **77**, 5877, 1955.

[16] E. Brand, W. H. Goldwater, and L. Zucker, Abstracts 106th meeting, Am. Chem. Soc., New York, September, 1944, p. 19B.

[17] In the analogous case of the ionization of the amino group in O-methyl tyrosine (D. B. Wetlaufer, J. T. Edsall, and B. R. Hollingworth, *J. Biol. Chem.* [in press]) it has been shown that the molar extinction coefficients are distinctly different for the forms $(-O^+)$ and $(-OO)$, in the region between 2750 and 2900 A. However, at 2950 and 3000 A, the extinction coefficients of both forms have fallen nearly to zero and the assumption of equality for the two ϵ_0 values introduces very little error in equation (12).

[18] R. G. Bates, *Electrometric pH Measurements* (New York: John Wiley & Sons, 1954).

[19] The preparation of O-methyl tyrosine was kindly provided by Professor H. T. Clarke and was twice recrystallized before use. The N-trimethyl tyrosine was prepared by one of us (R. B. M.); its preparation and properties will be described in detail in a later paper.

[20] A. Albert, *Biochem. J.*, **50,** 690, 1952. *Note:* Albert's values for tyrosine are: $pK_1 = 2.20$, $pK_2 = 9.19$, $pK_3 = 10.43$. Ionic strength not specified, but presumably low.

[21] C. Tanford and G. L. Roberts, Jr., *J. Am. Chem. Soc.*, **74,** 2509, 1952.

[22] The converse effect, of placing a negative charge on the hydroxyl group on the pK of the ammonium group (i.e. $pk_{13} - pk_{123}$), of course, gives the same result (see eq. [7]).

[23] E. J. Cohn and J. T. Edsall, *Proteins, Amino Acids and Peptides,* Chap. 6, p. 372.

[24] J. T. Edsall, G. Felsenfeld, D. S. Goodman, and F. R. N. Gurd, *J. Am. Chem. Soc.*, **76,** 3054, 1954.

[25] Y. Nozaki, F. R. N. Gurd, R. F. Chen, and J. T. Edsall, *J. Am. Chem. Soc.*, **79,** 2123, 1957.

---

*PTERIDINES IN PHOTOSYNTHESIS—AN ARTIFACT OF PAPER CHROMATOGRAPHY**

BY R. C. FULLER, I. C. ANDERSON, AND H. A. NATHAN

DEPARTMENT OF BIOLOGY, BROOKHAVEN NATIONAL LABORATORY, UPTON, N.Y.

Communicated March 24, 1958

In a previous issue of these PROCEEDINGS[1] we published a paper entitled "Pteridines in Photosynthesis." Further experiments to clarify the chemical nature of the C^{14}-labeled, yellow fluorescent compound described were undertaken, and it now has been shown that this compound with all the characteristics previously reported can be produced entirely outside the biological system. We had used hydroxylamine and phenylhydrazine to trap an apparently labile compound formed in less than 5 seconds of photosynthesis, using the method reported by Metzner *et al.*[2], in a number of plant species. The absorption spectrum of this compound when eluted from paper and its particular yellow fluorescence in long-wave-length ultraviolet light indicated a pteridine-type molecule. The radioactivity strikingly associated with this compound on paper was formed by the plant only in the light.

We have now found that both hydroxylamine and phenylhydrazine complex with an impurity in washed Whatman No. 1, No. 3, and No. 4 filter paper to give a yellow fluorescent compound that has an identical *Rf* in five solvents of the compound reported by us. The area on paper, when cut out and eluted, has an absorption spectrum with a strong peak at 285 mμ. This absorption is not present in the hydroxylamine in solution or when mixed with any of the five solvents used for chromatography. Nor is there a similar spectrum when hydroxylamine is complexed with several one-carbon compounds suspected of being the C^{14}-labeled fragment. Both the

fluorescence and the absorption spectrum are observed only after running the trapping agents on paper.

The close association of radioactivity with this compound on paper is yet to be explained. One explanation may be that there is an effect of salts on the mobility of C^{14}-labeled intermediates formed in short-term photosynthesis. Some experiments have indicated that when large amounts of the trapping agents are used, their salts "force" the compound into shapes and positions on the paper that are not representative of their true mobilities.

In any case, the presence of the short-term C^{14}-labeled fluorescent compound in photosynthesis reported by us as a pteridine is a result of the techniques employed in the experiments and is not a metabolic reality in photosynthesis.

* Research carried out at Brookhaven National Laboratory under the auspices of the U.S. Atomic Energy Commission.

[1] R. C. Fuller, I. C. Anderson, and H. A. Nathan, these PROCEEDINGS **44**, 239–244, 1958.

[2] H. Metzner, H. Simon, B. Metzner, and M. Calvin, these PROCEEDINGS **43**, 892–895, 1957.

---

*ON THE EMISSION OF TRYPTOPHAN**

BY IRVIN ISENBERG AND ALBERT SZENT-GYÖRGYI

INSTITUTE FOR MUSCLE RESEARCH, MARINE BIOLOGICAL LABORATORY, WOODS HOLE, MASSACHUSETTS

Communicated April 25, 1958

Introduction.—In a recent communication, Steele and Szent-Györgyi[1] reported the existence of two types of blue emission from tryptophan and from tryptophan-containing proteins. These two emissions, studied in frozen-water systems, were long-lived and relatively short-lived, respectively, the first resulting when the samples were excited at 280 mμ and the second when the samples were excited at 340 mμ.

The available evidence[2] indicates that the long-lived emission is a phosphorescence, a decay from a metastable triplet state. This report is an attempt to elucidate further properties of the short-lived emission. To this end, tryptophan emissions were studied in homogeneous glasses and in viscous supersaturated glucose solutions. The results indicate that the relatively short-lived emission comes from an association or interaction of tryptophan molecules and that the emission is from a metastable state.

Experimental.—Emission spectra were measured on a Beckman DK-1 recording spectrophotometer using the technique of Gemmill[3] and Steele and Szent-Györgyi.[1] The exciting light was isolated by means of a fused quartz Leiss Monochromator with a Hanovia high-pressure xenon arc (Model 10-C-1) as a source.

Most emissions were recorded with the sample dissolved in a supersaturated glucose solution, the concentrations of glucose ranging up to 21 gm/10 ml of water. The samples were prepared by dissolving the tryptophan in the water, bringing the solution almost to the boiling point and then dissolving the sugar. All tryptophan concentrations reported here refer to the final concentration of tryptophan in the

glucose solution. In the supersaturated glucose solutions, the glucose remains in solution for a number of hours. At the higher concentrations of sugar, 15–21 gm/10 cc water, the samples will form clear glasses when chilled to the temperature of dry ice.

The tryptophan used was the C.P. grade of L-tryptophan from the H. M. Chemical Company.

Results.—A short-lived blue emission was observed when tryptophan at 5×10^{-3} *M* to 10^{-2} *M* was excited by light in the region of 340 mμ. This emission increased both with increasing concentrations of tryptophan and with increasing concentrations of glucose. Figure 1 shows the effect of varying tryptophan concentrations; Figure 2 shows the effect of varying glucose concentrations. While, with our technique, accurate quantitative comparisons of emission intensity for dif-

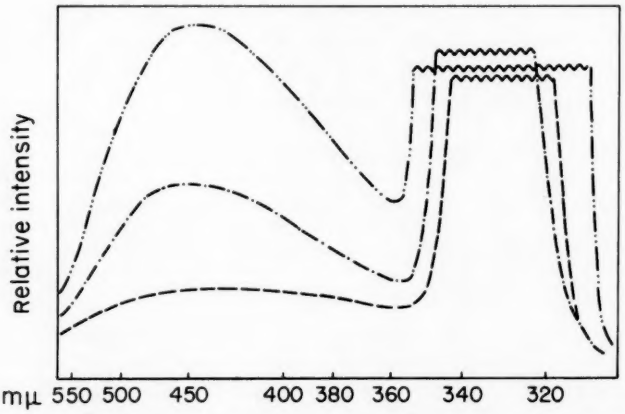


FIG. 1.—Emission of tryptophan in a glass at −78° C. The glass contained 21 gm. of glucose per 10 ml. of water. — — — 10^{-3} *M* tryptophan; — · — 5×10^{-3} *M* tryptophan; — · · — 10^{-2} *M* tryptophan. The broken peaks represent the exciting light.

ferent samples cannot be made, it was nevertheless clear that at 10^{-3} *M* tryptophan very little light was emitted in the glasses, while at 10^{-2} *M* tryptophan the light was quite intense. Similarly, at room temperature the light emitted from 10^{-2} *M* tryptophan was weak, though visible to the eye, with no sugar in the solution, and became much stronger with high concentrations of sugar.

Upon cutting off the exciting light, the blue emission at 470 mμ decayed too rapidly to be measurable with our apparatus.

Several samples of 10^{-2} *M* tryptophan in a glucose solution of 15 gm/10 cc were heated to about 80° C. and then placed in the sample holder in the Beckman housing. As the sample cooled to room temperature, the emission at 470 mμ increased markedly. It should be noted that at 80° C. such a solution is fluid, while at room temperature it is quite viscous.

Action spectrum for the short-lived emission were taken. These indicated that the emission was optimally excited in the vicinity of 360 mμ.

Discussion.—The short-lived blue emission of tryptophan and of tryptophan-containing proteins, investigated by Steele and Szent-Györgyi,[1] is evidently the same emission as that studied here in homogeneous solutions. This is indicated by three facts:

1. They are both excited in the same wave-length region. Steele and Szent-Györgyi reported a long-wave-length absorption shoulder at 340–350 mμ in the absorption curves for several tryptophan-containing proteins, while the band studied here is optimally excited at about 360 mμ.

2. They both emit in the same region; the band reported by Steele and Szent-Györgyi emits in the region of 420 to 470 mμ, depending on the protein studied, while the band studied here emits at 465 mμ.

3. They both have no afterglow that can be measured with the technique used.

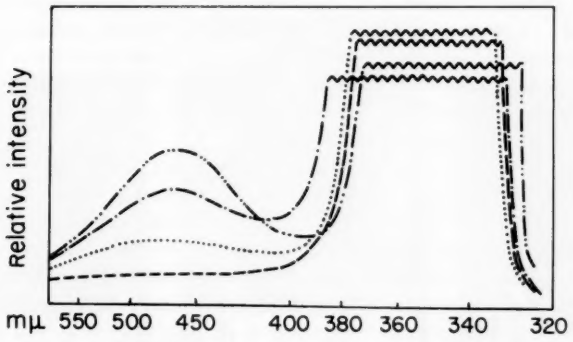


FIG. 2.—Room-temperature emission of 10^{-2} *M* tryptophan in solutions containing varying amounts of glucose. — — — no glucose, ... 5 gm. of glucose per 10 ml. of water; — · — 10 gm. of glucose per 10 ml. water; — · · — 15 gm. of glucose per 10 ml. of water.

The fact that the short-lived emission is strongly dependent on the concentration of tryptophan and that the emission is not characteristic of emission from individual tryptophane molecules suggests that tryptophan molecules interact in some way at high concentrations. The short-lived blue would then be the emission due to the interaction, resulting in an absorption band in the vicinity of 360 mμ.

The fact that the emission is dependent on sugar concentration and on temperature suggests that the emission is viscosity-dependent, which in turn indicates that the emission comes from a metastable state and is a forbidden transition rather than an allowed one.

* This research was sponsored by the grants from the National Heart Institute No. H-2042R. Commonwealth Fund, the National Science Foundation, the American Heart Association, the Association for the Aid of Crippled Children, and the United Cerebral Palsy Association.

[1] R. H. Steele and A. Szent-Györgyi, these PROCEEDINGS, **44**, 540, 1958.

[2] R. H. Steele and A. Szent-Györgyi, *ibid.*, **43**, 477–491, 1957.

[3] Gemmill, Ch. L., *Anal. Chem.*, **28**, 1061–1064, 1956.

ON DISSOCIATION AND RECOMBINATION OF HUMAN ADULT HEMOGLOBINS A, S, AND C

By Harvey A. Itano and S. J. Singer

NATIONAL INSTITUTES OF HEALTH* AND YALE UNIVERSITY†

Communicated by J. G. Kirkwood, April 14, 1958

INTRODUCTION

According to experiments reported by Field and O'Brien,[1] acidification of normal human adult hemoglobin (Hb A) from pH 6 to pH 3.5 is accompanied by a progressive, reversible decrease in sedimentation constant, together with an increase in translational diffusion constant. These findings were interpreted as showing the presence of a rapidly reversible, hydrogen-ion-dependent dissociation of Hb A into half-molecules. Vinograd,[2] using the more direct Archibald method of molecular weight determination,[3] has confirmed these results. It occurred to us that if other human hemoglobins also undergo reversible dissociation, "hybrid" molecules of hemoglobin might be formed by dissociation and reassociation of the subunits in a mixture of two different types of hemoglobin.

Our studies have dealt with Hb A and the abnormal adult forms, S (sickle cell) and C.[4, 5] Their comparative ultracentrifugal properties at different acid pH were found to be indistinguishable, indicating that the three types of hemoglobin are equally susceptible to reversible acid dissociation. Mixtures containing the three possible pairs of these types were acidified and neutralized. The sedimentation behavior of the neutralized samples showed that reassociation had occurred; however, electrophoretic analyses gave no indication of the presence of hybrid molecules.

MATERIALS AND METHODS

Preparation of Hb.—Blood samples of a normal adult and from subjects with sickle-cell anemia and hemoglobin C disease were obtained as sources of Hb A, S, and C, respectively. Following removal of plasma and white cells by centrifugation, the red cells were washed three times with 0.9 per cent NaCl solution and then lyzed by the addition of water and toluene.[6] A clear solution of oxyhemoglobin (HbO_2) was separated from the resulting mixture by centrifugation and converted by passage of carbon monoxide to the stock solutions of carbonmonoxyhemoglobin (HbCO) used in this work.

Preparation of Solutions.—For the ultracentrifuge experiments, the stock solutions of HbCO were diluted to 0.70 per cent with the appropriate buffer and were dialyzed at 4° C. against the CO-saturated buffer for 24 hours. A CO atmosphere was maintained in a closed vessel with magnetic stirring. However, overnight dialysis at pH 3.96 resulted in denaturation; therefore, solutions at this pH were examined immediately after preparation.

To investigate the recombination problem, two sets of experiments were performed independently in the two laboratories. In the first, mixtures of pairs of the appropriate stock HbCO solutions were made and diluted with cold, CO-saturated acetate buffer, pH 4.30, μ 0.1, to a total concentration of 0.80 per cent protein. After 1 hour at 0° C., these were dialyzed for 48 hours at 4° C. against the appro-

priate CO-saturated phosphate buffer used in electrophoresis. In the course of this dialysis a small amount of precipitate formed which was centrifuged down before electrophoresis. As controls, samples of the individual HbCO were subjected to the same acid treatment, were dialyzed back into the phosphate buffer near neutrality, and were then mixed to give the same over-all composition as their counterparts. The same small amount of precipitate developed in the individually acid-treated HbCO as in the mixtures and was discarded.

In the second set, 50-50 mixtures of pairs of the HbCO stock solutions were prepared at a total concentration of 2.5 per cent in water. Five ml. of each mixture was mixed with 5 ml. of acetate buffer, 0.2 *M* NaAc, 0.2 *M* HAc. The final pH was 4.7. After two hours at room temperature, 15 ml. of 0.1 *M* Na_2HPO_4 was added to bring the pH to 7.0. No precipitate formed. Controls were prepared by adding 20 ml. of phosphate buffer, pH 6.8, μ 0.1, to 5 ml. of each mixture. The

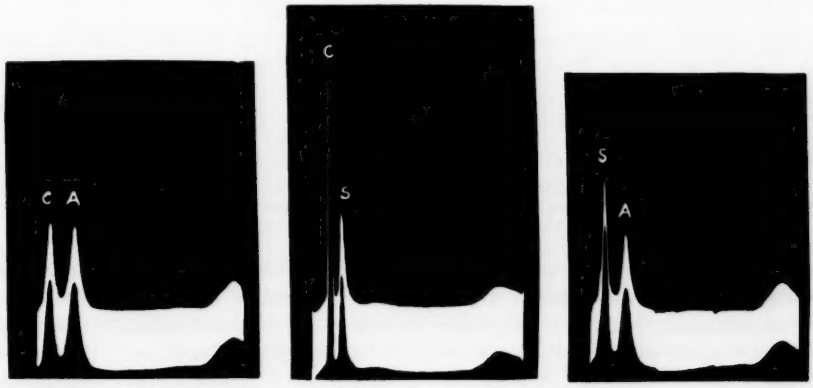


FIG. 1.—Electrophoretic patterns of mixtures of hemoglobin dissociated in pH 4.3, μ 0.1 acetate buffer and returned to neutral pH. First set of experiments discussed in text. At left, mixture of 58% HbA and 42% HbC, in phosphate buffer, pH 6.73, μ 0.018, after 4800 sec. at 19.8 volts/cm. In center, Mixture of 64 per cent HbS and 36 per cent HbC, in phosphate buffer, pH 6.95, μ 0.023, after 9600 sec. at 18.9 volts/cm. At right, mixture of 59% Hba and 41% Hbs, in phosphate buffer,p H 6.73, μ 0.018, after 6000 sec. at 19.8 volts/cm. All patterns are of the ascending limb.

final total protein concentration of each mixture was 0.5 per cent. Each sample was then treated with solid $Na_2S_2O_4$, resaturated with CO, and dialyzed into phosphate buffer, pH 6.8, μ 0.01 for electrophoresis.

Ultracentrifugation.—Experiments were performed in sodium acetate–acetic acid buffers at μ 0.10, in a Spinco Model E ultracentrifuge operated at 59,780 r.p.m. at temperatures near 25°. A Wratten No. 29 filter was interposed in the optical system, and type 103-F(3) spectroscopic plates were used.

Electrophoresis.—The first set of analyses, referred to above, was carried out in a Perkin-Elmer Model 38A Tiselius apparatus in buffers chosen to give adequate resolution. The conditions of the experiments are given in the legend of Figure 1. The second set of experiments was carried out in a Spinco Model H electrophoresis-diffusion instrument under conditions successfully employed for the resolution of intermediate compounds of HbCO and ferrihemoglobin.[7] A potential gradient of 9.1 volts/cm was applied for 14,400–18,000 seconds with a bath temperature of 0.5°.

RESULTS AND DISCUSSION

From the data of Table 1 it may be concluded that Hb A, S, and C dissociate indistinguishably in acid solution. On the other hand, the electrophoretic results (Fig. 1) have never disclosed any new components in acidified and reneutralized mixtures of these hemoglobins. Not only were the same two electrophoretic components present in the treated solutions as in the controls, but also the proportions remained unaltered. We may infer from these results that only certain specific modes of reassociation are possible. Our object in the following discussion is to relate these findings to the molecular structure of human adult hemoglobin. Preliminary to this discussion, however, we must first summarize some pertinent facts about hemoglobin.

TABLE 1

SEDIMENTATION OF HEMOGLOBINS

Hb	pH	S^{w}_{20}	pH	S^{w}_{20}	pH	S^{w}_{20}	pH	S^{w}_{20}	pH	S^{w}_{20}
A	6.73	4.08	5.65	3.81	4.79	2.79	4.30	2.49	3.96	2.38
A/S*	...	...	5.65	3.76	4.79	2.85	...	...	3.96	2.45
S	...	...	5.65	3.82	4.79	2.78	...	...	3.96	2.40
C	...	...	5.68	3.68	4.83	2.81	4.30	2.51	...	...
A + C†	6.73	4.19	...	...	...	...	...	...	...	...
S + C†	6.95	4.05	...	...	...	...	...	...	...	...

* A/S is hemoglobin, from sickle-trait individuals, containing Hb A + S.
† Mixture brought to pH 4.3, then returned to indicated pH, as described in text.

Some Aspects of the Structure of the Hemoglobin Molecule.—According to X-ray diffraction data, a molecule of HbO_2, of molecular weight *ca.* 66,000, possesses a dyad axis and is therefore composed of two apparently identical halves.[8] Chemical evidence exists which is also consistent with the identity of the halves. Recent results of Rhinesmith *et al.*[9] indicate that Hb A has four *N*-terminal valine residues on only two types of polypeptide chains. Furthermore, tryptic digestion[10] of Hb yields the number of peptides expected for a molecule containing a characteristic number of lysine and arginine residues in two identical halves. While these results do not entirely exclude the possibility that some small differences exist, we shall hereinafter assume that a particular Hb molecule consists of two identical halves and that each half is composed of two unlike polypeptide chains.

Hemoglobins A, S, and C were originally discovered to be distinct by virtue of small differences in electrophoretic mobility among them.[4, 5] However, the amino acid composition of Hb A and S is strikingly similar,[11] and the X-ray data for the two are indistinguishable.[8] The recent elegant studies of Ingram[10] have apparently explained the electrophoretic differences. In each half-molecule of Hb S a valine residue is present in a position occupied by a particular glutamic acid residue in Hb A. In Hb C the same glutamic acid residue is replaced in each half-molecule by a lysine residue.[12] It is very tempting to assume that these amino acid abberrations and perhaps some resulting structural dislocations in their immediate vicinity are the only abnormalities present in the molecules of Hb S and C. This hypothesis will be referred to as the "assumption of the localized defect." However, it is not impossible that other small differences also exist which have so far been undetected.

The Mode of Dissociation of the Hemoglobin Molecule.—The presence of a single

boundary in the sedimentation of partially dissociated preparations of Hb A has been ascribed to a rapid association-dissociation equilibrium between the parent molecule and its subunits.[13] If this be the case, one must consider the possibility that the half-molecules are also in rapid equilibrium with their constituent polypeptides. On the other hand, mean molecular weights of less than 33,000 have never been reported in dissociation experiments with Hb A, whether in acid or in mercaptoethanol and urea.[1, 14] Vinograd has found, moreover, that the mean molecular weight of partially dissociated Hb A in acid is consistent with the existence of an equilibrium between the intact molecule and two subunits of equal particle weight.[2] We conclude, therefore, that dissociation occurs into only two subunits.

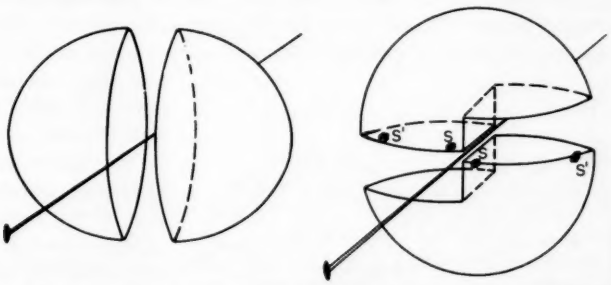


FIG. 2.—Two geometrically possible schematic modes of dissociation of the Hb molecule.

The geometry of this dissociation is at present undefined. Crystallographic investigations of HbO_2 A have shown that the dyad axis is along the *b*-direction and that five layers of polypeptide chains lie parallel to the long (*a*-direction) axis of the molecule. The dyad axis bisects the central layer of chains. One method of splitting the molecule which is consistent with these requirements was indicated by Bragg, Howells, and Perutz[15] and is schematically represented in Figure 2, *a*. However, a split roughly parallel to the long axis of the molecule (Fig. 2, *b*) is equally possible, as are numerous variations. In what follows, the surface along which the products of dissociation are actually joined in the parent molecule will be referred to as the "surface of contact."

If the Hb A molecule consists of identical halves, each half contains two polypeptide chains, which we may designate *a* and *u*. The intact molecule is then represented by the formula a_2u_2. There are two chemically different ways in which this molecule can dissociate: (1) into two identical units *au* and (2) into unlike units a_2 and u_2, each of which possesses dyad symmetry (Fig. 3). There is no a priori reason to consider either possibility more likely, particularly in view of the fact that Hb A contains no disulfide bonds[16] to link the polypeptide chains.

On the assumption of the localized defect in the Hb S molecule, its two polypeptide chains can be designated *s* and *u*, and the intact molecule represented by s_2u_2. If the assumption is correct, only that chain *s*, containing the valine instead of the glutamic residue in the Hb A polypeptide *a* is different, and the polypeptides *u* from Hb A and Hb S are identical. On the other hand, if other defects are also present in Hb S, a correspondingly slightly different polypeptide *v* can also exist, and in this

case Hb S can be described by the formula s_2v_2. Similar considerations apply to Hb C. As was just discussed in the case of Hb A, Hb S and Hb C can also conceivably dissociate into two identical, or into two unlike, subunits.

Structural Implications of the Absence of Hybrid Formation.—It is therefore evident that several different combinations of factors must be considered possible, depending on the nature of the differences in the polypeptide chains of Hb A, S, and C and the mode of splitting of the Hb molecule. Confining our attention to Hb A and S, we list the various possible alternatives in Table 2. Our finding that no hybrid hemoglobins are detectable electrophoretically eliminates those possibilities which should have produced such hybrids. The three remaining possibilities are: case 1, the defect in Hb S is not localized, and the reassociation of subunits from a particular type of Hb occurs specifically; case 2, the defect in Hb S is localized; the dissociation of Hb A produces identical subunits, and similarly for Hb S; and these subunits recombine only with their identical counterparts; or case 3, the defect in Hb S is localized; and the dissociation of Hb A produces the unlike subunits a_2 plus u_2, and, of Hb S, the units s_2 plus u_2.

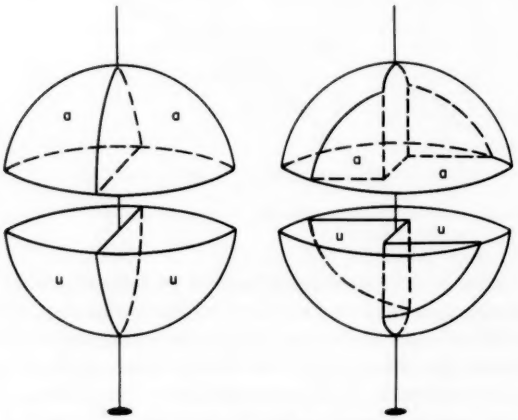


FIG. 3.—Two possible schematic modes of dissociation of Hb into two unlike subunits. The dyad axis is in the plane of the paper.

If the first case represents the true circumstances, our results in themselves can make no definitive contribution to the problem of Hb structure, and we shall therefore not discuss this case further.

If the second case is true and only identical subunits can recombine, such specificity implies that the localized defect of Hb S must occur at or near the surface of contact of the subunits. On the other hand, the abnormality of Hb S must also be responsible for the sickling phenomenon, which is the aggregation of intact Hb S molecules with one another in the absence of oxygen.[4, 17] It has been suggested[4] that this aggregation is due to the existence of complementary configurations on the deoxygenated Hb S molecule which form a bond with one another, somewhat analogous to that formed between antigen and antibody molecules. This implies, then, that the localized defect is on or near the exterior surface of the intact molecule. The two requirements are simultaneously satisfied if the defect occurs at or near the outer periphery of the surface of contact. There are two general positions which the defect might then occupy: (*a*) near the dyad axis, so that the defects of the identical half-molecules are in close proximity in the undissociated molecule (positions *S*, Fig. 2), and (*b*) away from the dyad axis, so that the defects behave independently (positions *S'*, Fig. 2). It may be readily seen that the positions *S'* are not con-

sistent with the assumption of a localized defect. If the symmetrically placed sites S' on a molecule of Hb S replace sites A on Hb A, and if these are the only differences between Hb A and Hb S, the same structure T must be opposite the sites S' and A in the undissociated molecules (Fig. 4, *I* and *II*). There would then be no reason

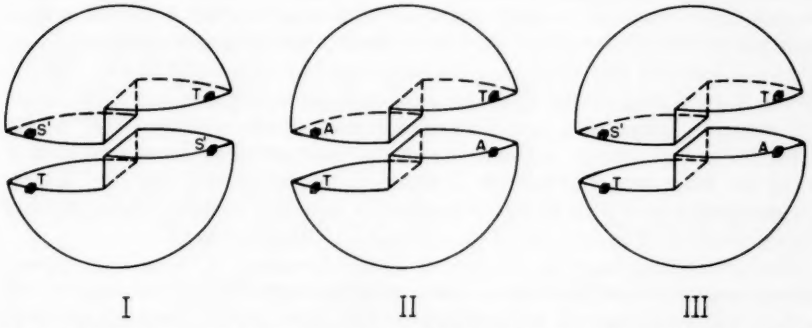


FIG. 4.—Schematic representations of HbS (*I*) and HbA (*II*) on the assumption of the localized defect in the region S'. The hybrid form (*III*) consists of a half-molecule of each.

to prevent the hybrid molecule (Fig. 4, *III*) from forming, since the sites A and S' in the hybrid would each be opposite the same structure T which they encounter in the respective parent molecules. The experimental fact is that hybrid electrophoretic species are not formed. Therefore, if the region of the known defect is a considerable distance (greater than about 5 A) from the dyad axis, the structure

TABLE 2

POSSIBLE MODES OF DISSOCIATION AND RECOMBINATION OF HB A AND S

Hb S Structure*	Nature of Dissociation	Remarks Concerning Recombination
s_2v_2	$\{a_2 + u_2\}$ $\{s_2 + v_2\}$	If homologous halves combine specifically, no hybrids detectable electrophoretically, no exchange occurs If heterologous halves can combine, four species should form, hybrids detectable electrophoretically if u_2 and v_2 bear different charges
	$\{au + au\}$ $\{sv + sv\}$	If homologous halves combine specifically, no hybrids detectable, no exchange occurs If heterologous halves can combine, three species should form, hybrids detectable electrophoretically
s_2u_2	$\{a_2 + u_2\}$ $\{s_2 + u_2\}$	No hybrids detectable electrophoretically, exchange should occur
	$\{au + au\}$ $\{su + su\}$	If homologous halves combine specifically, no hybrids detectable, no exchange occurs If heterologous halves can combine, three species should form, hybrids detectable electrophoretically

* The structure of Hb A is taken as a_2u_2.

complementary to it must also be different in the Hb A and S molecules; i.e., the assumption of a single localized defect cannot be correct. On the other hand, if the defect of Hb S is in fact localized to a single site, this site must exist within about 5 A from the dyad axis.

According to case 3, since the subunits u_2 derived from Hb A and Hb S are identical, reassociation can only produce the molecules a_2u_2 and s_2u_2, which are electro-

phoretically indistinguishable from the components of the original mixture. No electrophoretically distinguishable hybrids can form if s_2 and a_2 units cannot combine. However, exchange of u_2 units can occur, and this possibility can therefore be attacked experimentally by testing for such exchange. For example, a mixture of labeled Hb A and unlabeled Hb S can be subjected to the dissociation and reassociation conditions which we have described, the reassociated Hb A and Hb S fractionated, and their content of label determined. Assuming uniform labeling, we expect in this case that the fraction of label found in HbS would be $(M_{u_2}/M_{\mathrm{Hb}})f$, where M_{u_2} and M_{Hb} are the molecular weights of the u_2 fragment and of the intact Hb molecule, respectively, and f is the mole ratio of the unlabeled HbS to the total Hb in the original mixture. As in the previous case, the defect of Hb S must lie near the outer surface of the molecule to account for the sickling phenomenon, but its position with respect to the surface of contact is undetermined by our results. The extension of this discussion to Hb C is direct.

Genetic Implications of the Absence of Hybrid Formation.—The present experiments were stimulated by observations that hybrid molecules are not found in red cells of individuals who are heterozygous for the alleles which control the synthesis of Hb A, S, and C.[18] Each heterozygous cell contains only two of these types, A and S, A and C, or S and C. Is this absence of hybrid formation due to the biosynthesis of intact Hb A and S molecules, or are subunits synthesized and these, once in solution, may then associate only in specific combinations? Our experiments indicate that hybrids do not form even under favorable circumstances in solution. Therefore, it is not necessary to postulate the biosynthesis of intact Hb molecules, although this remains a possibility.

It is not known whether a single genetic locus controls the amino acid content and sequence of a half-molecule or whether different loci control the two polypeptide chains of each half-molecule. In either case, if the subunits are released from the site of synthesis as the asymmetric monomers, *au* and *su* in the first case or *a*, *s*, and *u* in the second, we may postulate that the same structural factors discussed earlier are responsible for the specific combination of subunits and the absence of hybrid molecules. Either the defect of *su* or *s* is not localized, or, if it is localized, it occurs near the dyad axis of the complete molecule. Another possible explanation for the absence of hybrids is that dimerization occurs at the site of biosynthesis. If a pair of alleles correspond to the half-molecules, *au* and *su*, the products released from the site of biosynthesis would be the symmetrical molecules, a_2u_2 and s_2u_2. If different loci control the two types of polypeptides in each molecule and if the defect is localized to one of the peptides, the dimers a_2 and s_2 would result from a pair of alleles at one locus, and only u_2 would result from the homozygous second locus. These dimers could combine into the two molecules, a_2u_2 and s_2u_2. Double heterozygosity would result in four dimers, a_2, s_2, u_2, and v_2, potentially capable of combining into a_2u_2, a_2v_2, s_2u_2, and s_2v_2. Genetic test of the latter type of interaction is not yet available because the observed aberrations of Hb S and Hb C both occur in the same polypeptide chain.

We are indebted to Mrs. Ruth E. Singer for performing many of the experiments reported in this paper and to Drs. J. R. Vinograd and E. P. Geiduschek for simulating discussions.

Note added in proof: In order to avoid confusion, uniformity in the nomenclature of the Hb chains is desirable. Before such uniformity can be achieved, however, it will be necessary to determine which N-terminal sequence[9] corresponds to the chain which carries the localized defect.

* National Institute of Arthritis and Metabolic Diseases, Public Health Service, U.S. Department of Health, Education, and Welfare, Bethesda, Maryland.

† Chemistry Department, Yale University, New Haven, Connecticut, Contribution No. 1482.

[1] E. O. Field and J. R. P. O'Brien, *Biochem. J.*, **60,** 656, 1955.

[2] J. R. Vinograd, paper presented at the meeting of the American Chemical Society, New York, September 8–13, 1957.

[3] S. M. Klainer and G. Kegeles, *J. Phys. Chem.*, **59,** 952, 1955.

[4] L. Pauling, H. A. Itano, S. J. Singer, and I. C. Wells, *Science*, **110,** 543, 1949.

[5] H. A. Itano and J. V. Neel, these PROCEEDINGS, **36,** 613, 1950.

[6] D. L. Drabkin, *J. Biol. Chem.*, **164,** 703, 1946.

[7] H. A. Itano and E. Robinson, *J. Am. Chem. Soc.*, **78,** 6415, 1956.

[8] M. F. Perutz, A. M. Liquori, and F. Eirich, *Nature*, **167,** 929, 1951.

[9] H. S. Rhinesmith, W. A. Schroeder, and L. Pauling, *J. Am. Chem. Soc.*, **79,** 4682, 1957.

[10] V. M. Ingram, *Nature*, **178,** 792, 1956; **180,** 326, 1957.

[11] W. A. Schroeder, L. M. Kay, and I. C. Wells, *J. Biol. Chem.*, **187,** 221, 1950.

[12] V. M. Ingram, presented at Symposium on Protein Structure and Biological Specificity, Paris, July 25–29, 1957; see K. Bailey, *Nature*, **180,** 833, 1957.

[13] E. O. Field and A. G. Ogston, *Biochem. J.*, **60,** 661, 1955.

[14] F. J. Gutter, H. A. Sober, and E. A. Peterson, *Arch. Biochem. Biophys.*, **62,** 427, 1956.

[15] W. L. Bragg, E. R. Howells, and M. F. Perutz, *Acta Cryst.*, **5,** 136, 1952.

[16] R. E. Benesch, H. A. Lardy, and R. Benesch, *J. Biol. Chem.*, **216,** 663, 1955.

[17] J. W. Harris, *Proc. Soc. Exptl. Biol. Med.*, **75,** 197, 1950.

[18] H. A. Itano, *Advances in Protein Chem.*, **12,** 215, 1957.

---

*EFFECT OF AGE ON THE ENZYME ACTIVITY IN ERYTHROCYTES**

BY PAUL A. MARKS, ANNE B. JOHNSON, AND ERICH HIRSCHBERG

DEPARTMENTS OF MEDICINE AND BIOCHEMISTRY, COLUMBIA UNIVERSITY COLLEGE OF PHYSICIANS AND SURGEONS

Communicated by Robert F. Loeb, April 18, 1958

INTRODUCTION

The destruction of human red blood cells, under normal circumstances, is related to senescence.[1] This observation suggests the possibility that the aging of erythrocytes and their eventual disintegration may result from a progressive diminution in the activity of certain metabolic systems essential to the maintenance of the integrity of these cells. This investigation was designed to determine whether alterations in the activity of enzymes occur in associated with the in vivo aging of mature red blood cells.

It has been demonstrated by other investigators that the activities of cholinesterase glyoxalase, and catalase are elevated in the human erythrocyte populations containing a high percentage of reticulocytes.[2–4] In addition, Allison and Burn[4] found that cholinesterase and catalase levels fall progressively in transfused donor cells as they age in vivo. Recently, the activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase, but not of purine nucleoside phos-

phorylase, were found to be high in erythrocytes of patients with an increased proportion of young red blood cells.[5]

The enzymes assayed in the present investigation include glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase. These dehydrogenases catalyze reactions providing the only known mechanism in mature red blood cells for oxidation of glucose to CO_2 with the generation of reduced triphosphopyridine nucleotide. In addition, phosphohexose isomerase and lactic acid dehydrogenase were studied. These two enzymes are involved in glycolysis via the Embden-Meyerhof pathway, the major source of energy in erythrocytes. Purine nucleoside phosphorylase, which catalyzes the phosphorolytic cleavage of purine nucleosides to ribose-1-phosphate and purine, has also been assayed.

In the present experiments, samples of erythrocytes with relatively young and old mean cell ages were obtained, employing techniques based on the fact that young mature red blood cells are more resistant to hypotonic lysis[6] and that they are less dense.[7, 8] Enzymatic assays performed on such samples of erythrocytes of differing mean cell age have revealed that glucose-6-phosphate dehydrogenase, 6-phosphogluconic dehydrogenase, and phosphohexose isomerase, but not lactic acid dehydrogenase or purine nucleoside phosphorylase, activities decrease markedly with the aging of red blood cells in vivo.

MATERIALS AND METHODS

Fifteen ml. of heparinized venous blood was drawn from subjects in good health. All blood samples were centrifuged,[9] and the plasma and buffy coat removed. The erythrocytes were washed with isotonic potassium chloride buffered at pH 7.4 and resuspended in this solution so as to provide a hemoglobin concentration of approximately 150 mg/ml. This procedure was performed at 0° C. These resuspended washed erythrocytes will be referred to as the "whole erythrocyte population." Aliquots of the whole erythrocyte population were removed for counting erythrocytes, leukocytes, and reticulocytes. Erythrocytes completely free of leukocytes and reticulocytes could not be obtained. It was found, however, that the activities of the enzymes per gram of hemoglobin were unaffected, provided that the contamination of erythrocytes by leukocytes and reticulocytes was less than 0.05 and 0.5 per cent, respectively.

Separation of Young and Old Erythrocytes.—The whole erythrocyte population was separated into fractions of relatively young and old mean ages by two different methods: (*a*) serial osmotic hemolysis and (*b*) centrifugation.

a) *Serial osmotic hemolysis:* One-ml. samples of the whole erythrocyte population were added to a tube containing 5 ml. of distilled water and to a series of tubes containing 5 ml. of sodium chloride solutions of varying degrees of hypotonicity. The tubes were mixed and allowed to stand at 0° C. for 10 minutes. The samples were centrifuged, and the supernatant solutions were removed and saved. The sedimented unlysed erythrocytes were washed promptly with 10 volumes of ice-cold isotonic potassium chloride buffered at pH 7.4 and resuspended in about 3 ml. of this solution. These erythrocyte samples and an aliquot of the whole erythrocyte population were hemolyzed by freezing and thawing twice.

The concentration of the sodium chloride solutions (usually 0.33–0.46 per cent sodium chloride) employed were empirically chosen to cause hemolysis of approxi-

mately 2, 5, 10, 90, 95, and 98 per cent of the added erythrocytes. The per cent hemolysis achieved by a given hypotonic sodium chloride solution was expressed as the ratio of the hemoglobin concentration in the supernatant solution to the hemoglobin concentration in the solution of water-lysed erythrocytes, multiplied by 100.

Serial osmotic hemolysis achieves a separation of mature human erythrocytes into cell populations of relatively young and old mean cell ages. This was established by determining the concentration of erythrocytes of varying ages, as dated by Fe^{59} labeling in vivo, in the fractions of cells of different osmotic resistance.[10] It was found that the 10, 5, and 2 per cent least resistant cells were progressively more enriched with regard to old erythrocytes and impoverished with respect to young and middle-aged red blood cells. The unlysed cells of the 90, 95, and 98 per cent levels of hemolysis were progressively more enriched with young red cells and impoverished with respect to old and middle-aged erythrocytes. The supernatant solutions of the 2 ± 1, 5 ± 1, and 10 ± 1 per cent levels of hemolysis and the sedimented cells of the 90 ± 1, 95 ± 1, or 98 ± 1 per cent levels of hemolysis were chosen for assay of enzyme activities. These erythrocyte fractions will be referred to as the 2, 5, and 10 per cent least resistant and 10, 5, and 2 per cent most resistant cells, respectively. In addition, enzyme assays were performed on the water-lysed cells and the whole erythrocyte population.

b) Centrifugation: Six ml. of the whole erythrocyte population were centrifuged in 10×150-mm. tubes for 30 minutes at 2,000 *g*. The upper, middle, and lower 15 per cent of the packed column of erythrocytes were removed and hemolyzed by freezing and thawing twice. Enzyme assays were performed on these hemolyzates. It has been shown, employing Fe^{59} to date the age of erythrocytes, that the upper, middle, and lower layers of centrifuged human red cells have young, intermediate, and old mean cell ages, respectively.[8]

Enzyme Assays.—Glucose-6-phosphate dehydrogenase was assayed by a method based on that of Kornberg and Horecker;[11] 6-phosphogluconic dehydrogenase by a technique similar to that of Horecker and Smyrniotis;[12] and purine nucleoside phosphorylase by a modification of the method of Price *et al.*[13] The details of these enzyme assays have been described elsewhere.[5]

Phosphohexose isomerase was determined according to the method of Bodansky,[14] and lactic acid dehydrogenase by a procedure based on the technique of Hill and Levi.[15] The activities of glucose-6-phosphate dehydrogenase, 6-phosphogluconic dehydrogenase, purine nucleoside phosphorylase, and lactic acid dehydrogenase were expressed as the change in optical density per minute per gram of hemoglobin (ΔOD/min/gmHgb). A unit of phosphohexose isomerase activity was defined as the reciprocal of that amount of sample, expressed as grams of hemoglobin per milliliter reaction mixture, which, under the standard conditions of the assay, produced 25 μg. fructose as fructose-6-phosphate from glucose-6-phosphate. Hemoglobin concentrations were measured by pipetting a suitable quantity of hemolyzate into 10 ml. of 0.4 per cent NH_4OH and reading the optical density of the resulting solution in a spectrophotometer at 540 mμ.

Enzyme Recovery Studies.—To determine whether the fractionation of the whole erythrocyte population by serial osmotic hemolysis affected enzyme activity, the total activity of each enzyme was measured in the supernatant solution and sedimented cells of the various fractions. The sum of the enzyme activity in the

supernatant solution and that in the sedimented cells expressed as per cent of the enzyme activity in the whole erythrocyte population revealed the following mean values ± one standard deviation:

For glucose-6-phosphate dehydrogenase: 98.2 ± 5.3 per cent;
For 6-phosphogluconic dehydrogenase: 102.2 ± 5.3 per cent;
For purine nucleoside phosphorylase: 97.0 ± 5.1 per cent;
For phosphohexose isomerase: 101.8 ± 6.9 per cent;
For lactic acid dehydrogenase: 96.1 ± 6.3 per cent.

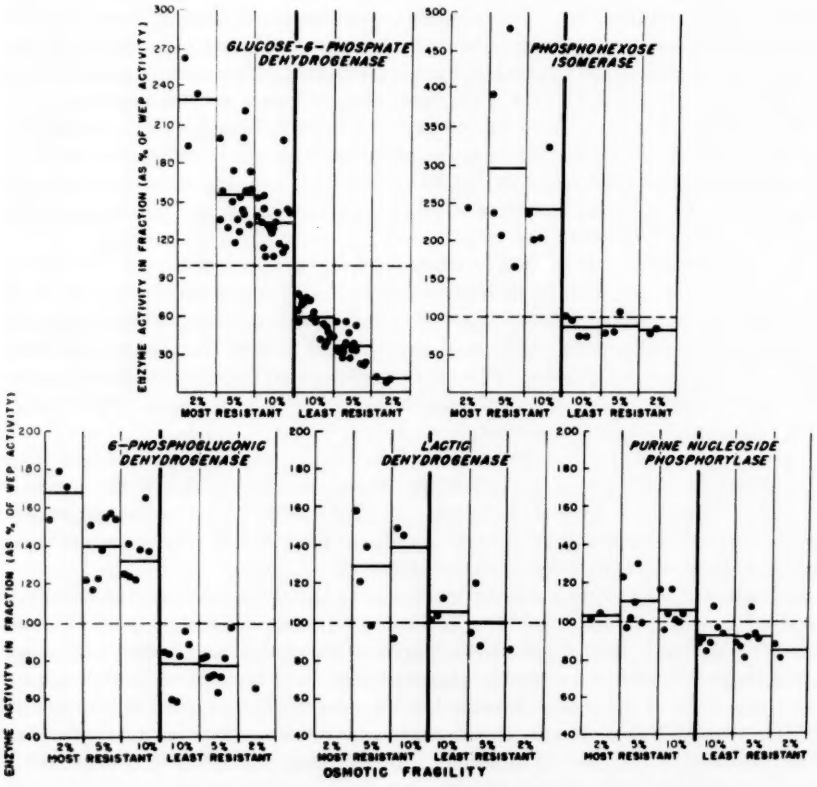


FIG. 1.—Enzyme activity in erythrocyte fractions of varying osmotic fragility. This figure contains all the data obtained for enzyme activity in erythrocytes fractionated by serial osmotic hemolysis. For each enzyme, the activity found in the various fractions (2, 5, and 10 per cent least resistant and 10, 5, and 2 per cent most resistant fractions) is expressed in terms of the per cent of the activity of the whole erythrocyte population (WEP). The cross-bars indicate the mean values. These data represent 22 studies for glucose-6-phosphate dehydrogenase, 6 studies for phosphohexose isomerase, 11 studies for 6-phosphogluconic dehydrogenase, 5 studies for lactic dehydrogenase, and 9 studies for purine nucleoside phosphorylase.

There were no significant differences in activities of these enzymes in hemolyzates prepared by freezing and thawing compared with hemolyzates prepared by water

lysis. In addition, partially purified preparations of glucose-6-phosphate dehydrogenase,[11] 6-phosphogluconic dehydrogenase,[12] and purine nucleoside phosphorylase[16] added to hemolyzates of various fractions gave theoretical values within the analytical error of the enzymatic essays.

RESULTS

Enzyme Activity in Erythrocytes Fractionated by Serial Osmotic Hemolysis.—In red blood cells fractionated by serial osmotic hemolysis, the activities of glucose-6-phosphate dehydrogenase, 6-phosphogluconic dehydrogenase, and phosphohexose isomerase were markedly higher in the more resistant than in the less resistant erythrocyte fractions. Purine nucleoside phosphorylase and lactic acid dehydrogenase levels were reduced only 20–40 per cent in the least resistant cell fractions (Fig. 1).

TABLE 1

ENZYME ACTIVITY IN ERYTHROCYTE FRACTIONS OF VARYING OSMOTIC FRAGILITY: ILLUSTRATIVE EXAMPLES OF SINGLE FRACTIONATION FOR EACH ENZYME

			Fraction of Erythrocytes†					
			Most Resistant (Per Cent)			Least Resistant (Per Cent)		
Enzyme*	Subject	WEP*	2	5	10	10	5	2
G-6-P. D.	LZ	16.3	38.3	26.1	23.2	7.3	3.9	1.7
6PG. D.	DE	20.7	35.4	32.1	...	17.0	14.8	...
PHI	ZA	14.2	...	68.0	46.0	10.7	...	11.3
PNP	LI	173	179	166	178	145	139	138
LDH	PE	141	...	154	161	124	133	...

* Abbreviations include: G-6-P. D., glucose-6-phosphate dehydrogenase; 6PG. D., 6-phosphogluconic dehydrogenase; PHI, phosphohexose isomerase; PNP, purine nucleoside phosphorylase; LDH, lactic acid dehydrogenase; WEP, whole erythrocyte population.

† Units of enzyme activity are: G-6-P. D., 6PG. D., PNP and LDH: Δ OD/min/gm Hgb; PHI: 10^3 units/gm Hgb.

The most resistant and least resistant erythrocyte fractions have relatively younger and older mean cell ages, respectively. Accordingly, the enzyme activities may be related directly to the relative mean age of the red blood cells in the various fractions. Among different subjects, there was an overlap in the range of enzyme activity for erythrocyte fractions of differing mean cell age (Fig. 1). This variation may be attributed in part, to the fact that erythrocyte fractions of a given osmotic fragility prepared from red cell populations of different subjects varied in the degree of relative enrichment with young and old cells. However, in a given fractionation study, it was consistently observed that the older the relative mean cell age of the erythrocyte sample, the lower the activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase (Table 1).

Of the five enzymes studied, glucose-6-phosphate dehydrogenase activity showed the most marked difference between the most and least resistant red blood cell fractions. The mean values for enzyme activity in the 5 per cent most resistant compared to the 5 per cent least resistant erythrocyte fractions differed by a factor of 4.0 for glucose-6-phosphate dehydrogenase, 3.3 for phosphohexose isomerase, 1.9 for 6-phosphogluconic dehydrogenase, 1.2 for lactic acid dehydrogenase, and 1.2 for purine nucleoside phosphorylase. Comparing enzyme activity in the young cell fractions with that in the whole erythrocyte population, the decrease in phosphohexose isomerase was most striking (Fig. 1).

Enzyme Activity in Erythrocytes Fractionated by Centrifugation.—The levels of

glucose-6-phosphate dehydrogenase, 6-phosphogluconic dehydrogenase, and phosphohexose isomerase, but not of purine nucleoside phosphorylase, were higher in the upper, less dense, than in the lower, more dense, erythrocyte fraction (Table 2). Among individuals there was again an overlap in the range of these dehydrogenase activities between the least and the most dense erythrocyte fractions. For a given erythrocyte fractionation study, however, the activities of glucose-6-phosphate dehydrogenase, 6-phosphogluconic dehydrogenase, and phosphohexose isomerase were consistently higher in the less dense red blood cells.

The fractions of erythrocytes of relatively young and old mean cell ages separated by centrifugation showed smaller differences in glucose-6-phosphate dehydrogenase, 6-phosphogluconic dehydrogenase, and phosphohexose isomerase activities than did the fractions of differing mean cell age prepared by serial osmotic hemolysis. These observations with regard to enzyme activity are consistent with the finding based on Fe^{59} specific activity that, of the two methods employed in this study, serial

TABLE 2

ENZYME ACTIVITY IN ERYTHROCYTE FRACTIONS OF VARYING DENSITY

FRACTION OF CELLS*	ENZYME ACTIVITY†			
	ΔOD/MIN/CM HGB			PHI (10 UNITS/ GM HGB)
	G6P. D.	6PG. D.	PNP	
WEP	17.3 (12.8–21.9)	14.2 (12.1–16.4)	144 (135–160)	16.2 (14.2–18.2)
Top 15 per cent cells	20.3 (14.4–24.7)	16.7 (13.1–19.8)	145 (136–158)	19.2 (16.1–22.3)
Bottom 15 per cent cells	14.9 (11.9–19.0)	13.0 (10.6–16.4)	141 (124–154)	12.9 (10.7–14.1)

* WEP represents the whole washed erythrocyte population. The top 15 per cent cells and bottom 15 per cent cells refer to the upper and lower 15 per cent of a column of packed erythrocytes (see text).

† The values for the enzyme activity represent the mean and range (indicated in parentheses) of 15 studies for G6P. D., 3 studies for 6PG. D., 5 studies for PNP, and 2 studies for PHI. Abbreviations are as defined in n.* of Table 1.

osmotic hemolysis achieved a more effective fractionation of the whole erythrocyte population.

DISCUSSION

The present study indicates that the levels of certain enzymes in mature human erythrocytes decrease with the aging of these cells in vivo. The activities of glucose-6-phosphate dehydrogenase, 6-phosphogluconic dehydrogenase, and phosphohexose isomerase fall more markedly than do those of purine nucleoside phosphorylase and lactic acid dehydrogenase. In addition, among these enzymes, differences were apparent in the pattern of this decline in activity. Thus the level of glucose-6-phosphate dehydrogenase was progressively lower, the older the red cell fraction, while phosphohexose isomerase activity decreased primarily in the younger cell fractions. It must be emphasized that the differences between young and old red blood cells with respect to the activities of these enzymes are very much greater than the differences measured in the fractions of cells of varying osmotic resistance. These fractions are merely enriched with young or old erythrocytes.

It is of interest that the decrease in enzyme levels with erythrocyte aging in vivo are in striking contrast to the lack of change in enzyme activity in cells stored in vitro. It has been found that red cells stored at 4° C. in acid-citrate-dextrose solu-

tion, or acid-citrate-dextrose-inosine solution for as long as 7 months showed no change in the levels of glucose-6-phosphate dehydrogenase- 6-phosphogluconic dehydrogenase, purine nucleoside phosphorylase, or phosphohexose isomerase.[17]

The explanation of the decline in enzyme activity with erythrocyte aging in vivo is not apparent from our present knowledge. A possible interpretation of these observations is that, following the loss of nucleic acids, the erythrocyte cannot synthesize proteins. The previously synthesized complement of enzymes may then fall progressively as the cell becomes older. There is good evidence that the mature erythrocyte cannot synthesize heme.[18] The data with regard to their ability to synthesize stromal proteins are inconclusive.[19] There have been no studies on enzyme synthesis in mature red blood cells. If mature erythrocytes do indeed synthesize enzymes, then the diminution in activity of enzymes with age may be related to alterations in rates of turnover of these proteins. These problems are being investigated.

Evidence has accumulated to indicate that the maintenance of the integrity of the erythrocyte membrane is dependent on various enzymatically catalyzed reactions.[20] In view of the present finding that the levels of certain erythrocyte enzymes are related to the age of these cells, it is reasonable to suggest that the diminution in activity of critical enzymes may determine the life-span of the red blood cell in vivo.

SUMMARY

The activities of certain enzymes have been studied in mature human erythrocytes of relatively young and old mean cell ages. The samples of young and old red blood cells were separated by methods based on the fact that young, compared to old, erythrocytes are less osmotically fragile and are less dense. The activities of glucose-6-phosphate dehydrogenase, 6-phosphogluconic dehydrogenase, and phosphohexose isomerase decrease more markedly with the aging of red blood cells in vivo than do those of lactic acid dehydrogenase and purine nucleoside phosphorylase. It is suggested that the decline in activity of critical enzymes is a determinant of the life-span of erythrocytes.

* Supported in part by grant C2332 of the National Cancer Institute. A portion of the presen data was presented before the Society for Clinical Investigation, Atlantic City, N.J., May, 1957 and appeared in abstract form: P. A. Marks, *J. Clin. Invest.*, **36**, 913, 1957.

The authors are indebted to Maureen Donohue and Stanley Goldstein for their assistance in the course of these studies.

[1] D. Shemin and D. Rittenberg, *J. Biol. Chem.*, **166**, 627, 1946.

[2] J. C. Sabine, *J. Clin. Invest.*, **19**, 833, 1940.

[3] L. M. Meyer. A. Sawitsky, M. D., Ritz, and H. M. Fitch, *J. Lab. Clin. Med.*, **33**, 189, 1948.

[4] A. C. Allison and G. P. Burn, *Brit. H. Haemat.*, **1**, 291, 1955.

[5] P. A. Marks, *Science* (in press).

[6] D. Chalfin, *J. Cell. Comp. Physiol.*, **47**, 215, 1956.

[7] J. C. Dreyfus, G. Schapira, and J. Kruh, *Compt. rend. Soc. de biol.*, **144**, 792, 1950.

[8] E. R. Borun, W. G. Figueroa, and S. M. Perry, *J. Clin. Invest.*, **36**, 676, 1957.

[9] All centrifugations were carried out at 1,500 *g.* for 10 minutes, unless otherwise noted.

[10] P. A. Marks and A. B. Johnson (in preparation).

[11] A. Kornberg and B. L. Horecker, in S. P. Colowick and M. O. Kaplan, *Methods in Enzymology* (New York), **1**, 323, 1955.

[12] B. L. Horecker and P. Z. Smyrniotis, *J. Biol. Chem.*, **193**, 371, 1951.

[13] Y. E. Price, M. C. Otey, and P. Plesnes. in S. P. Colowick and M. O. Kaplan, *Methods in Enzymology* (New York), **2**, 448, 1955.
[14] O. Bodansky, *Cancer*, **7**, 1191, 1954.
[15] B. R. Hill and C. Levi, *Cancer Research*, **14**, 513, 1954.
[16] K. K. Tsuboi and P. B. Hudson, *J. Biol. Chem.*, **224**, 879, 1957.
[17] P. A. Marks A. B. Johnson R. DeBellis, and J. Banks, *Fed. Proc.*, **17**, 269, 1958.
[18] I. M. London, D. Shemin, and D. Rittenberg, *J. Biol. Chem.*, **183**, 749, 1950.
[19] H. M. Muir, A. Neuberger, and J. C. Perrone, *Biochem. J.*, **52**, 87, 1952.
[20] E. Ponder, *Blood*, **9**, 227, 1954.

SPONTANEOUS NUCLEATION IN SUPERSATURATED VAPORS

BY WORTH H. RODEBUSH AND JOHN V. CLARKE, JR.

W. A. NOYES LABORATORY, UNIVERSITY OF ILLINOIS, URBANA

Communicated April 15, 1958

At the end of the last century C. T. R. Wilson[1, 2, 3, 4] established that a given degree of supersaturation was required to produce homogeneous nucleation in a vapor. The technique used was the adiabatic expansion of the vapor in the presence of a non-condensable gas, and the test for the nucleation was the instantaneous (or very rapid) formation of a visible cloud of liquid droplets. Wilson recognized that some ions were always present to contribute to the nucleation, and the principal use of the cloud chamber soon came to be a method of making ion tracks visible. Various experimenters have tried to remove ions from the chamber by electric fields. The most satisfactory method of reducing the number of ions present, as judged by our experience with the maximum degree of supersaturation obtainable, is by the use of clean apparatus and a gas such as hydrogen or helium, which contains a small number of electrons per molecule and contributes a small amount of intrinsic ionization.

Spontaneous nucleation must be regarded as superposed on ionization, and its observation must therefore be indirect, depending on such factors as the rate of growth of the cloud of droplets in density. The density of the cloud, however, depends very much upon the critical drop size, which may be defined as the size of drop which gives the maximum vapor pressure at a given temperature. The existence of such a critical drop size is demonstrated by an equation for quasi-thermodynamic equilibrium derived by one of the authors.[5] The equation contains two terms which are functions of drop radius. One contains the free surface energy which tends to make the drop unstable, and the other expresses the stabilizing effect of the entropy of the drop as a polyatomic molecule of gas. The two opposing terms lead to a maximum for the free energy of the drop as a function of radius. So long as the critical size is large, the drop containing 100 molecules or more, the equation is quite exact. When the drop size is small—for example, in the case of mercury because of the large surface tension of mercury—the concept of the free surface energy of the drop calculated as a sphere becomes very approximate indeed.

In order to test the application of this equation, studies were made upon the supersaturation of a number of liquids of different surface tensions and vapor pressures.

In Wilson's first work[1] the critical supersaturation value of 4.2-fold at 0° C. was found to hold for many gases. However, the value for water vapor in hydrogen was found to be above 5-fold. Wilson correctly ascribed ths result to the relatively small number of naturally occurring ions in hydrogen.

In this laboratory the condensation of water vapor in helium was found to require supersaturations of above 6-fold. Here again the low degree of ionization is undoubtedly responsible for the failure to achieve condensation at the well-known figure of 4.2-fold. From this it is obvious that the electric fields used in so many of the investigations did not remove more than a portion of the ions present. It is equally obvious that the supersaturation values measured with ion-removing fields are not values for spontaneous nucleation but apply only to droplet formation assisted by ions.

Although most cloud-chamber work has been aimed at ionization detection, a few investigators have devoted their efforts to fundamental condensation processes. In a series of experiments Volmer[6, 7] proved conclusively the temperature dependence of the critical supersaturation. Using a strong electric field to remove ions, the critical point for water vapor was found to be approximately 4.9-fold near $-10°$ C., slightly higher than Wilson's value. Just beyond the point at which the first scattered droplets appeared, Volmer noted that a slight increase in supersaturation gave rise to a marked increase in the number of droplets. He appears to have regarded this point of increase as the onset of spontaneous nucleation. His experimental results have been widely used as a test for theoretical calculations of the spontaneous critical point.

Sander[8] and Powell[9, 10] have done similar work on water vapor. Sander's results are lower than those of most other workers (e.g., 3.7-fold near 0° C.). Although he employed an ion-clearing field, his method of saturation may have produced charged aggregates which would grow at lower supersaturations.

Aside from this last case, the experimental results of all the investigations have been in good agreement. There is little doubt that the reported supersaturation valves are correct; but the interpretation of the results as to "spontaneous" nucleation is in question.

The recent work of Pollermann,[11, 12] who determined "spontaneous" formation of drops on ions by measuring the supersaturation at which ion-induced droplets grew to such an extent that they no longer moved in a powerful field, is also open to question. It would appear that, instead of spontaneous formation on ions, the measured supersaturation was more likely that for rapid growth of previously formed charged aggregates. His reported value is 5.3-fold for formation on negative ions as compared to Volmer's 4.1-fold.

Much of the previously reported condensation work has involved water vapor. Because of its strong polarity and unusual structure in the liquid state, water must be a unique liquid. In order to study spontaneous nucleation, it seemed desirable to use the non-polar vapor of mercury and the relatively weakly polar benzene. In addition, to determine the effect of highly soluble impurities such as might normally be present in "pure" water, condensations were caused in the vapors above aqueous solutions containing mannitol, sodium chloride, and glycerol.

In order to cause condensation, the vapor being studied was supercooled by an adiabatic expansion caused by the sudden opening of the vessel containing the vapor

to an evacuated 10-liter flask. The final temperature attained during an expansion was calculated by thermodynamic formulas in terms of initial and final pressures assuming adiabatic conditions.

Because of the low vapor pressure of mercury at room temperature, visual detection of condensation was not feasible. Instead, a sensitive ultraviolet photometer was constructed which could follow the concentration of mercury vapor during an expansion by measuring the absorption of 2537 A ultraviolet radiation. Purified nitrogen and helium were bubbled slowly through glass frits into liquid mercury contained in a series of gas-washing bottles located in a constant-temperature bath. The equilibrium mixture of mercury vapor and inert gas was led into a quartz-windowed 3-liter flask. The initial concentration of mercury vapor could be calculated from the temperature of the liquid mercury in the saturator, which had to be kept a degree or so below room temperature to prevent the mercury from condensing out on the walls of the expansion chamber. During an expansion the concentration of mercury vapor was followed with the photometer to detect any indication of condensation. The steepness of the light absorption versus concentration curve for mercury vapor indicated that even the formation of bimolecular clusters would have a pronounced effect on the light-absorption measurements.

Expansions as large as could be made with the apparatus produced no indication of condensation. At the limit of the region in which the expansion can be considered to be nearly adiabatic, a final temperature of $-25°$ C. and a supersaturation of slightly over 100-fold were calculated. Using helium as the inert gas, the largest expansions which could be produced gave a calculated final temperature of approximately $-200°$ C. It is, of course, impossible to hope to reach any such temperature. Assuming that the final temperature did go as low as $-50°$ C., the resulting supersaturation would have been close to 1,000-fold.

Since the photoelectric detector could not cope with higher concentrations of mercury vapor, it was necessary to employ a visual detection method. Mercury vapor contained above hot liquid mercury in a heated flask was pulled into the large cool neck of the flask during an expansion and observed there in a powerful light beam. Since the flask neck was near room temperature, a very large temperature drop was obtained. It was discovered that visible clouds were formed when the initial temperature of the mercury was above 100° C. The cloud density increased as the temperature was raised, up to about 150° C., and then decreased with further rise in temperature. At an initial temperature of 134° C., an expansion going from 1 atmosphere down to approximately 8 cm. was required to produce condensation. A final pressure difference of 0.5 cm. was the range between dense condensation and no visible condensation.

Using helium, the calculated final temperature was approximately $-174°$ C., again far too low to be reached. Since the portion of the flask in which condensation took place was at approximately 29° C., it seems reasonable to assume that a final temperature of near $-25°$ C. was attained. The calculated supersaturation at this final temperature is approximately 10^4-fold. It is worthy of note that all of the condensation observed was truly cloudlike, similar to cigar smoke in appearance. There was no trace of the droplets or coarse mist which is observed near the onset point in other vapors.

Using the values

$$
\begin{aligned}
S^{\circ} &= 100 \text{ eu} \\
\gamma &= 441 \text{ dynes/cm} \\
V_L &= 14.8 \text{ cm}^3\text{/mole,} \\
S^{\circ} &= 100 \text{ eu,} \\
T &= 253^{\circ} \text{ K.,}
\end{aligned}
$$

the Rodebush equation predicts a critical supersaturation of 10^7-fold for mercury vapor, with a critical cluster of five atoms and 3×10^{-8} cm. radius. Calculation shows that insertion of a surface-tension value of 300 dynes/cm leads to a predicted supersaturation of 10^4-fold, in agreement with the experimental results.

Benzene vapor and helium supercooled above extremely pure liquid benzene, condensed into a regular spray of 1 or 2 droplets/cc at supersaturations of approximately 8-fold near -25° C. The value of the heat capacity ratio, k, for the mixture of helium and benzene was calculated, using the formula of Richarz,[14]

Using the values

$$
\begin{aligned}
S^{\circ} &= 100 \text{ eu} \\
\gamma &= 34.7 \text{ dynes/cm,} \\
T &= 250^{\circ} \text{ K.,} \\
V &= 88.6 \text{ cm}^3\text{/mole,}
\end{aligned}
$$

the Rodebush equation predicts a critical supersaturation of 8.3-fold, in excellent agreement with experimental results.

Water vapor and helium above pure water forms a thin regular spray of about 1 droplet/cm^3 at about 7-fold supersaturation near -8° C. The apparatus was tested using water vapor and nitrogen, and it duplicated Volmer's[8] figure of 4.1-fold near 0° C. Since helium with its low ion content requires higher supersaturations to cause condensation than air or nitrogen with an ion-clearing electric field, it appears obvious that the fields used removed only a portion of the ions present. Condensation observed under those circumstances was therefore not truly spontaneous but involved formation on the remaining ions.

The Rodebush equation predicts a value of about 4-fold near -10° C. for water vapor, while the experimental results indicate that spontaneous nucleation occurs somewhere between 6-fold and 9-fold, where the number of particles formed far exceeds the number of ions present.

Using a mixture of helium and water vapor above solutions containing mannitol, glycerol, and sodium chloride, the effect of impurities upon the critical point was investigated. Mannitol at concentrations up to 1.0 M had no apparent effect upon the condensation. Glycerol at 0.0001 M reduced the critical point from the 7-fold value for pure water to 5-fold. Sodium chloride at 0.001 M had no effect, while 1.0 M NaCl raised the critical point to 9-fold, even after allowing for the vapor pressure lowering effect of the solute. Pollermann[12] and Loeb[13] have reported a similar increase in the critical point upon the addition of HCl to the vapor. In all the calculations, the value of the heat-capacity ratio of the gaseous mixture was calculated, using the formula of Richarz.[14]

Summarizing the experimental results, we find that the experimental and theoretical values for the critical point in water and benzene are in good agreement. Here the critical cluster is calculated to be around 40 molecules for benzene and 100

for water. For mercury with a critical size cluster of under 10 atoms, the equation has lost its validity.

[1] C. T. R. Wilson, *Proc. Roy. Soc. London*, **189**, 265, 1897.
[2] C. T. R. Wilson, *Phil. Trans.*, **A192**, 189, 1899.
[3] C. T. R. Wilson, *Ibid.*, **A193**, 289, 1900.
[4] C. T. R. Wilson, *Phil. Mag.*, **7**, 681, 1904.
[5] W. H. Rodebush, these PROCEEDINGS, **40**, 789, 1954.
[6] M. Volmer and H. Flood, *Z. physik. Chem.*, **A170**, 273, 1934.
[7] M. Volmer and G. Tohmfor, *Ann. Physik*, **33**, 109, 1938.
[8] A. Sander and G. Damkohler, *Naturwiss.*, **31**, 460, 1943.
[9] C. F. Powell, *Proc. Roy. Soc. London*, **A119**, 553, 1928.
[10] C. F. Powell, *Engineering*, **127**, 799, 1929.
[11] M. Pollermann, *Ann. Physik*, **5**, 329, 1950.
[12] M. Pollermann, *Z. Electrochem.*, **56**, 313, 1952.
[13] L. B. Loeb, A. F. Kip, and A. W. Einarsson, *J. Chem. Phys.*, **6**, 264, 1938.
[14] F. Richarz, *Ann. Physik*, **19**, 457, 1908.

---

*STUDIES ON THE EXCITED STATES OF PROTEINS**

BY RICHARD H. STEELE† AND ALBERT SZENT-GYÖRGYI‡

INSTITUTE FOR MUSCLE RESEARCH, MARINE BIOLOGICAL LABORATORY, WOODS HOLE, MASSACHUSETTS

Communicated March 30, 1958

INTRODUCTION

If a bullock lens is illuminated by near UV, it emits a brilliant blue light, which is due to its proteins.[1] This is unexpected because two of the three aromatic amino acids—phenylalanine and tyrosine—have no visible fluorescence, and tryptophane has but a very weak one. A similar blue light is emitted if the lens is cooled in dry ice or liquid N_2. In neither case does the lens show an afterglow. If, however, the lens is illuminated by shorter UV, then a long-lasting afterglow can be observed. The monomolecular kinetics of the decay indicate that the light, in this case, was emitted from a metastable triplet state.[2] In order to arrive at a better understanding of these phenomena, we studied the optical properties of the aromatic amino acids and a number of proteins better defined than the proteins of the lens. Our results can be summed up by saying that the optical behavior of the lens and other proteins, under our conditions, is dominated by their tryptophane and that tryptophane, in a frozen watery solution, also shows two emissions—a short-lived one elicited by near UV and a long-lived one elicited by a shorter wave length. This indicates that the tryptophane within the protein is present in two different states which can be identified by their different excitability and light emission. The analogous two states can be detected also in frozen watery solutions of this amino acid.

EXPERIMENTAL

Absorption spectra were measured with the Beckman DK-1 automatic recording spectrophotometer. A variable-path quartz absorption cell was used in the absorption-spectra measurements of the concentrated protein preparations.

Room-temperature emission spectra of the proteins were determined with the Beckman DK-1 spectrophotometer, using the technique described by Gemmill.[3] For low-temperature emission studies (77° K.) this technique was modified by positioning an unsilvered Dewar flask containing liquid nitrogen in the DK-1 lamp housing compartment. The samples under study were placed in quartz test tubes and precooled in an alcohol dry-ice bath and then inserted into the liquid nitrogen. Exciting light was supplied by a Hanovia high-pressure xenon arc (Model 10-C-1). The wave-length region for excitation was isolated with a Leiss single-prism (fused-quartz) monochromator. It should be recognized that, with the exceptions noted below, none of the emission spectra for proteins and the detailed study of trypto-

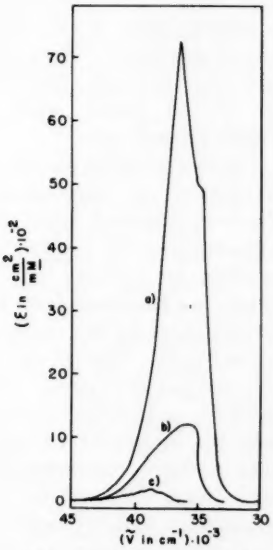


FIG. 1.—Molar extinction wave numbers for the aromatic amino acids: *a*, tryptophane; *b*, tyrosine; *c*, phenylalanine.

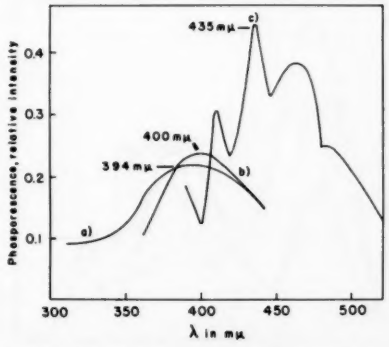


FIG. 2.—Phosphorescent spectra of (*a*) phenylalanine, (*b*) tyrosine, (*c*) tryptophane. Aqueous 0.001 *M* solutions containing 0.5 per cent glucose. $T = 77°$ K.

phane were corrected for the spectral-response sensitivity of the photomultiplier detector in the DK-1 spectrophotometer or for the prism efficiency of the monochromator of the DK-1. This uncorrected feature applies as well to the transmitted exciting light curves. It is to be noted, however, that the photomultiplier was an RCA 1P28 multiplier tube with S-5 response[4] with peak sensitivity at approximately 340 mμ. In practice, on the short-wave-length side of the 340-mμ maximum, the recorded curves (including the transmitted exciting light curves) are shifted approximately 5 mμ toward longer wave lengths, while on the long-wave-length side of 340 mμ the recorded curves are shifted approximately 5 mμ toward shorter wave lengths. These shifts are not important for any of the conclusions reported in this paper.

The fluorescent and phosphorescent spectra reported in Figure 2 were obtained

using Farrand grating monochromators to isolate the exciting light (xenon arc) and to analyze the emitted spectra. These curves were corrected for the spectral sensitivity of the RCA 1P28 photomultiplier tube.

RESULTS

Aromatic Amino Acids.—Figure 1 gives the absorption spectra of the three aromatic amino acids which are responsible for most of the 2300–3100 A absorption of protein. There is no apparent absorption at a longer wave length than 32,000 cm.$^{-1}$ (3120 A). The longest-wave-length absorption of tryptophane appears to be the unresolved peak masked under the shoulder at 34,000 cm.$^{-1}$ (2,900 A).

Our measurements of the fluorescent emission of these amino acids are in good agreement with those of Teale and Weber,[5] the maxima being at 284, 302.5, and 348 mμ for phenylalanine, tyrosine, and tryptophane, respectively. These emissions represent transitions from the lowest excited singlet state to the singlet ground state.[6]

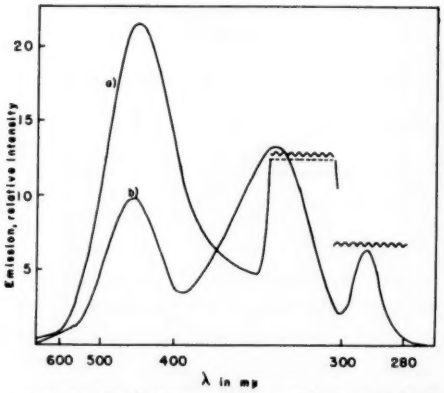


FIG. 3.—Emission spectrum of intact bullock lens, (*a*) excited in the 340-mμ region; (*b*) excited in the 280-mμ region. In all figures the wave line marks transmitted exciting light.

Figure 2 gives the phosphorescent spectra for the three amino acids. We have presented data[2] which led us to interpret the low-temperature emission for tyrosine and tryptophane as a transition from the lowest excited triplet state to the singlet ground state. Though the phenylalanine emission decay was too rapid for us to make kinetic studies with the equipment we had available, we have interpreted the long-wave-length shift with its close correspondence to the tyrosine emission maximum as evidence that this emission is also triplet-singlet in character.

PROTEINS

Bullock Lens.—At room temperature, excitation in the general region of the 280-mμ protein absorption band gave rise to an emission with a maximum at 340 mμ which we consider to be fluorescence originating from tryptophane residues in the protein. Shifting the exciting light to longer wave lengths elicited an intense emission with the same blue color as the phosphorescence we had observed from the same preparation at low temperature (77° K.).

Curve *b* in Fig. 3 gives the emission spectra of the bullock lens excited within the protein 280-mμ absorption band and by near UV at 77° K. (liquid N_2). The 330-mμ emission band had no delayed emission observable visually on our not too fast instruments. The 450 mμ band, in contrast, exhibited a considerable afterglow.

When we first observed the intense blue emission elicited by excitation in the 340-mμ region, we suspected that we might be inducing a direct singlet-triplet transi-

tion from the ground state or else that we were exciting the protein in a heretofore undetermined absorption band.

It might be reasoned that a singlet-triplet transition which would give rise to the intense emission we observed should manifest a reasonably strong absorption and that, consequently, a spectral examination of a concentrated protein preparation might reveal this fact. Such a study did, indeed, reveal a long-wave-length maximum in the absorption spectrum at 340 mμ. A long-wave-length shoulder was observed in this general region for all the proteins we have examined to date, but we have not felt justified in publishing the data, for we have not assured ourselves that scattering was not considerable in our experiments. Suffice it to point out that other workers, notably Goodwin and Morton,[7] have noted an "irrelevant absorption" in proteins in the 340–400-mμ region, where the aromatic amino acids do not absorb. When, however, the lens was excited in this absorption region at 77° K., the intense blue emission had no prolonged decay time. We may conclude that, if the low-temperature, long-wave-length emission elicited in the 340-mμ region was originating from a metastable triplet level, it was not the same level that is populated under the same conditions by exciting the protein in the 280-mμ absorption band.

Homogenized lens exhibited the same spectral behavior as did the intact material. The addition of glucose to the lens homogenate did not influence the lifetime of the 77° K. blue emission elicited by excitation in the long-wave-length 340-mμ region.

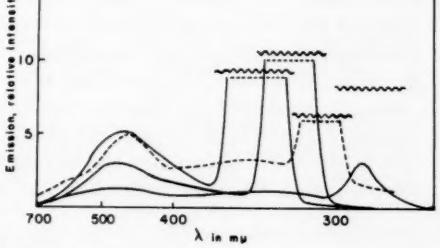


FIG. 4.—Emission spectrum of bovine serum albumin, excited by light of different wave lengths. 10 per cent aqueous solution, containing 1 per cent glucose. T = 77° K.

Albumin, Trypsin, Myosin.—Spectral data for bovine serum albumin, recrystallized trypsin, and myosin are represented in Figures 4–6. The emissions elicited by irradiation in the main protein absorption band around 280

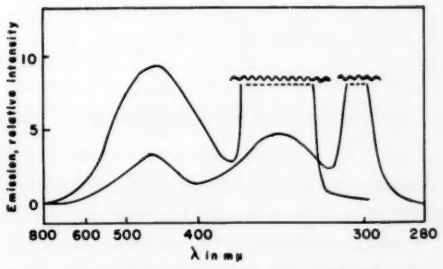


FIG. 5.—Emission spectra of recrystallized trypsin (0.245 gm. ml.$^{-1}$). T = 77° K.

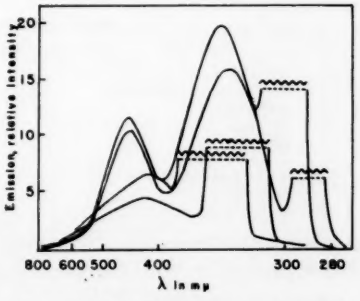


FIG. 6.—Emission spectra of myosin at different exciting wave lengths. 0.24 per cent aqueous solution. T = 77° K.

mμ were long-lived showing a considerable afterglow, while the emissions elicited by near UV around 340 mμ were short-lived. No afterglow could be seen visually or detected by our relatively slow instrumentation. The results are summed up in Table 1.

With the exception of the long-wave-length emission of myosin, elicited by exciting in the 340-mμ region, the emission spectra for the several proteins mirror the emission properties of tryptophane more than that of phenylalanine or tyrosine. Parenthetically, the close similarity between the low-temperature emission properties of the proteins and tryptophane, elicited by exciting in the absorption bands, supports the concept expressed by Teale and Weber[5] and ourselves,[8] that energy absorbed by phenylalanine or tyrosine is transferred to tryptophane, from which molecule emission occurs. The minimum in the emission spectra around 400 mμ, where phenylalanine and tyrosine phosphoresce (see Fig. 2), also bears out this idea. It might be considered that the high molar absorbancy of tryptophane relative to that of tyrosine or phenylalanine would result in tryptophane being the primary emitter. An examination of Table 2, where the molar ratios of the aromatic amino acid residues in the proteins are given, shows that, when allowance is made for the ratio of molar absorbancies (approximately 1:5:35 for phenylalanine, tyrosine, and tryptophane, respectively; see Fig. 1), phenylalanine and tyrosine should be almost as efficient emitters as tryptophane. Further, when the quantum yields are considered, tyrosine has approximately the same value as tryptophane, namely, 0.21 and 0.19, respectively (Teale and Weber[5]). The yield for phenylalanine is only 0.045, which may account for the nonobservance of an emission in the 300-mμ region, where phenylalanine and tyrosine fluoresce. Conversely, it may be

TABLE 1*

Protein	Long-Wave-Length Absorption in Aqueous Solution	Fluorescence 298° K.	Fluorescence 77° K.	Long-Lived Phosphorescence	Emission Elicited by Near UV (≧ 340 mμ) 298° K.	Emission Elicited by Near UV (≧ 340 mμ) 77° K.
Bullock lens	340	345	335	450	440	440
Bovine serum albumin	350	340	335	460	470	470
Trypsin	340	342	337	450	450	450
Myosin	340?	340	333	440	?	420

* Numbers stand for wave lengths of maxima in mμ.

TABLE 2*

MOLES OF AMINO ACIDS IN 10^5 GM. PROTEIN

Protein	Phenylalanine	Tyrosine	Tryptophane
Bullock lens	49	35	11
Bovine serum albumin	39	29	3
Trypsin	10	23	5
Myosin	28	18	4
Insulin	52	69	0

* compiled from date taken from R. J. Block and K. W. Weiss, *Amino Acid Handbook* (Springfield, Ill.: Charles C Thomas, 1956).

argued that the low quantum yield of fluorescence is evidence for an increased transition probability to the triplet state, where it may be more readily degraded as heat due to the increased lifetime. We, however, are inclined to the idea that the excitation energy is transferred by resonance to tryptophane and then emitted.[8]

Insulin and Tryptophane.—The close similarity in the emission properties of the

proteins and tryptophane led us to a comparative study of the emission behavior of tryptophane and of insulin, which contains no tryptophane.

Since we have previously noted[2] that the blue *delayed* emission of tryptophane (phosphorescence) was observable only after the addition of glucose to the system (in the absence of glucose what appeared to be the same blue emission was short-lived and decayed too rapidly for us to measure the decay kinetics), we examined the emission behavior with and without glucose. Though the kinetic behavior bore out our earlier observations, the emission spectra revealed interesting new details. These studies with watery solutions of tryptophane-no-glucose and tryptophane-glucose gave the following results:

1. Tryptophane-no-glucose excited in the absorption band at 77° K. displayed no fluorescent emission band, and the blue emission band with maximum at 435 mμ had no afterglow.

2. In the absence of glucose, 340-mμ excitation, at 77° K., elicited 435-mμ emission, just as found for proteins; this emission also had no afterglow.

3. Tryptophane *in the presence of glucose* at 77° K. exhibited a fluorescent band with a maximum at 325 mμ, and the blue band emitted at 440 mμ displayed a phosphorescent afterglow.

4. Long-wave-length excitation of tryptophane *in the presence of glucose* failed to elicit the 435-mμ short-lived emission. In these experiments aqueous 10^{-4} *M* tryptophane solutions were used.

The insulin was studied at 77° K., and the exciting light was varied from 260 to 450 mμ. When the protein was excited in the region of protein absorption (260–290 mμ), there was evidence of emission in the 290–340-mμ region, which was suggestive of fluorescence from the phenylalanine and tyrosine residues, but the intensity was too low to make positive assertion. It is of interest to note, however, that we observed no 440-mμ emission band, no matter what exciting wave-length region was selected. That is, phosphorescence, elicited by exciting in the region of protein absorption, was absent, as was the short-lived long-wave-length emission elicited by exciting the protein in the 340-mμ region. Insulin contains no tryptophane.

* This work was supported, in part, by grants from the National Institutes of Health (RG 4643 and RG 5374).

† Present address: Department of Biochemistry, School of Medicine, Tulane University, New Orleans 18, Louisiana.

‡ This work was supported, in part, by a grant from the Commonwealth Fund, the National Heart Institute (H-2042R), the Muscular Dystrophy Associations of America, Inc., the National Science Foundation, the American Heart Association, the Association for the Aid of Crippled Children, and the United Cerebral Palsy Foundation.

[1] Szent-Györgyi, A., *Biochim. et Biophys. Acta*, **16**, 167, 1955.

[2] Steele, R. H., and Szent-Györgyi, A., these PROCEEDINGS, **43**, 477–91, 1957.

[3] Gemmill, Ch. L., *Anal. Chem.*, **28**, 1061–64, 1956.

[4] Radio Corporation of America, *Tube Handbook*.

[5] Teale, F. W. J., and Weber, G., *Biochem. J.*, **65**, 476–82, 1957.

[6] For all spectra reported in this paper (with the exception of that reported in Fig. 2) quartz monochromators were used to isolate both exciting and emitted spectral regions, and nondescript transmission was no problem.

[7] Goodwin, T. W., and Morton, R. A., *Biochem. J.*, **40**, 628–32, 1946.

[8] Karreman, G., Steele, R. H., and Szent-Györgyi, A., these PROCEEDINGS (in press).

WALL THICKENING IN SIEVE ELEMENTS

By Katherine Esau and Vernon I. Cheadle

DEPARTMENT OF BOTANY, UNIVERSITY OF CALIFORNIA, DAVIS, CALIFORNIA

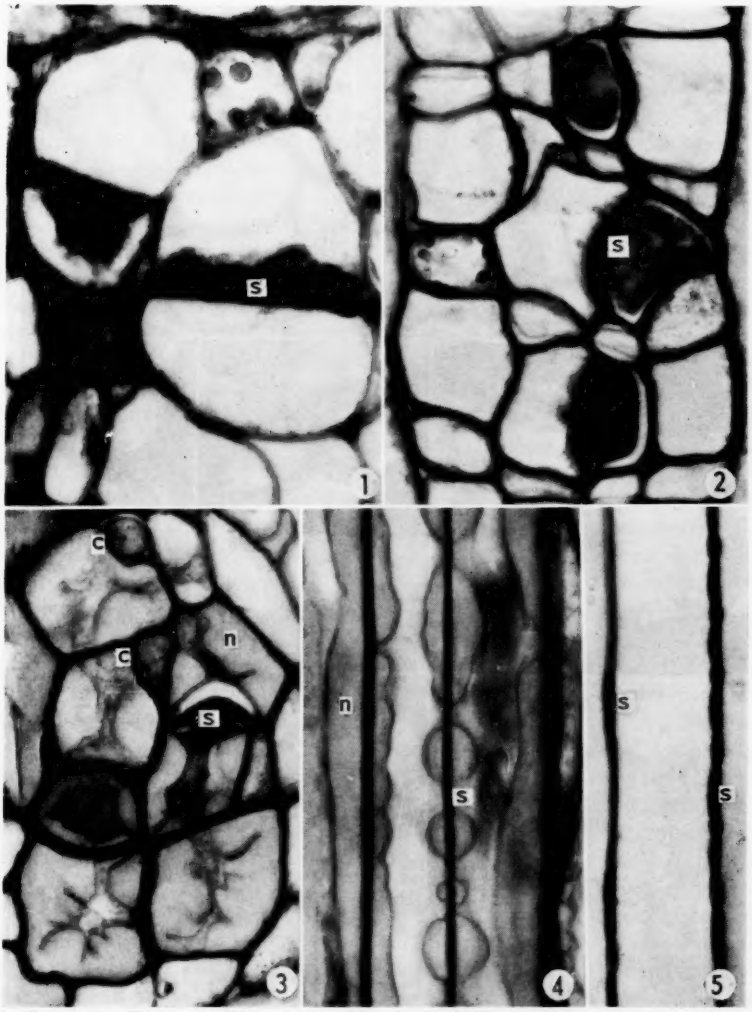
Read before the Academy, April 28, 1958

Because sieve elements are generally regarded as the specialized conducting cells in the phloem tissue, their structure in relation to function has been the subject of many studies and discussions.[1] Both the protoplast and the walls of sieve elements have distinctive characteristics, some of which are difficult to relate to the rapid flow of materials—at the rate of up to 100 cm. per hour—recorded in the phloem tissue. We have recently stressed one such feature[2] not previously observed: in many dicotyledons having relatively long cambial initials, various anticlinal divisions of the cambial derivatives result in sieve elements shorter than such initials. Thus reduction in the potential length of the sieve elements introduces additional end walls, and the moving solution must pass through a greater number of sieve plates. The present study is concerned with another feature that would seem to interfere with a free flow of materials through the sieve elements—the remarkably thick walls that occur in sieve elements of many species of dicotyledons.

The material included 142 species in 121 genera of 58 families of dicotyledons. In 67 per cent of the species, each was represented by more than one collection, usually two, one taken during dormancy, the other during the early or late part of the growth season. Part of the collections was made in California, part in Rhode Island. Only secondary phloem was examined, mostly from branches over ten years old. The methods of preparation of material for microscopic study were the same as described by Cheadle and Esau.[3] To obtain some notion of the relation of wall thickness to cell size, the sieve elements and their lumina were measured in transections along their tangential and radial diameters, 10 measurements of each for each plant. The cell diameters were measured from middle lamella to middle lamella, the lumina from wall to wall. The difference between the diameter of the cell and that of the lumen, divided by 2, gave the thickness of the wall. The product of the tangential and radial diameters was used to estimate the transectional area of the sieve element and of its lumen.

It should be emphasized that these measurements and calculations have only a rough comparative value. The material used was in killed condition, and in such material the walls are somewhat thinner than in fresh sections. Further, the transections of sieve elements and their lumina are not rectangular (see figures), and thus the products of tangential and radial diameters give only approximate size values of these areas.

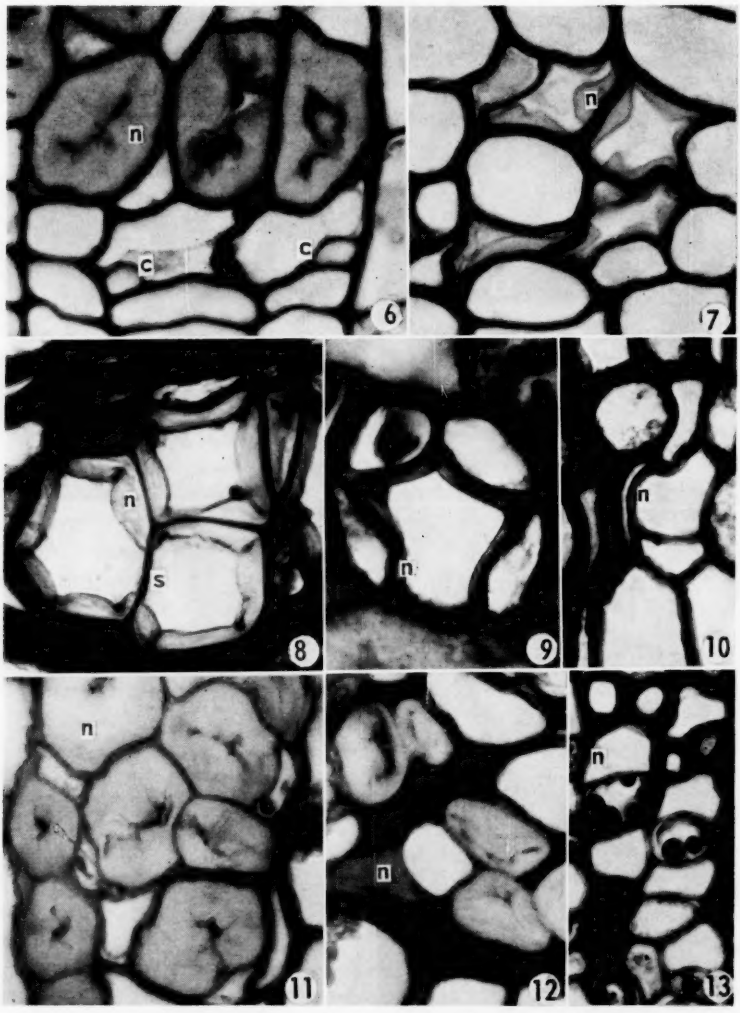
The walls of sieve elements of different species show a wide range of variations in thickness. Some species have walls approximately 1 μ thick (Figs. 1, 2, 5); in others the wall almost occludes the lumen of the cell (Figs. 3, 11). Many intermediate thicknesses occur in various other species (e.g., Figs. 7, 8). There is also a variation in the appearance of the walls. In some species the walls seem to be rather homogeneous (Figs. 1, 2, 5); even the middle lamella may not be distinguish-



FIGS. 1–5.—Transverse (Figs. 1–3) and longitudinal (Figs. 4, 5) sections of secondary phloem from stems, comparing sieve elements without nacreous walls (Figs. 1, 2, 5) and those with such walls (Figs. 3, 4). Fig. 1, *Juglans regia* L., collected November 15, 1950; Figs. 2, 5, *Populus grandidentata* Michx., collected July 24, 1951; Figs. 3, 4, *Magnolia kobus* DC., collected July 18, 1951. Details are: *c*, companion cell; *n*, nacreous wall; *s*, sieve area. All ×850.

able. In others the wall consists of two parts: a relatively thin layer next to the middle lamella and a more or less thick inner layer next to the lumen (Figs. 3, 4, 6, 7, 8, 11). This inner layer is the subject of the present report.

We have previously called the inner wall thickening of sieve elements "nacreous wall"[3] because the term "nacreous," mostly in its French equivalent *nacré*, has long been used in the literature on phloem to indicate the glistening appearance of



FIGS. 6–13.—Transverse sections of secondary phloem from stems, comparing nacreous walls in presumably functional sieve elements (Figs. 6–8, 11, 13) and those no longer functional (Figs. 9, 10, 12). The figures also illustrate variations in thickness of the nacreous wall. In Fig. 6 the immature sieve elements below (with companion cells at *c*) are free of nacreous walls. Fig. 6, *Magnolia kobus* DC., collected July 18, 1951; Fig. 7, *Laurelia novae-zealandiae* A. Cunn., collected May 29, 1951; Figs. 8–10, *Liriodendron tulipifera* L., collected May 10, 1951; Figs. 11, 12, *Talauma* sp., collected February 1, 1951; Fig. 13, *Kalmia latifolia* L., collected December 8, 1951. Details are: *c*, companion cell; *n*, nacreous wall; *s*, sieve area. All ×850.

sieve-element walls in fresh sections. The authors who introduced this term also described the nacreous walls as being rather thick and specified that the thickening reached its maximum development shortly before, or upon completion of, the dif-

ferentiation of sieve elements. Most of the old literature on nacreous wall thickening pertains to primary phloem.[4]

Although the term "nacreous" is not sufficiently specific—other phloem cells, and thick primary walls in general, commonly glisten in fresh sections—it is used in this paper as a term of convenience. Specifically, it is applied only to the inner layer of the wall if this layer is morphologically distinct from the layer deposited earlier, that is, the outer layer of the wall. "Nacreous wall," "nacreous wall thickening," "nacreous thickening," "nacreous layer," "inner wall," and "inner wall thickening" are used synonymously.

When striking differences in wall thickness occur among sieve elements, these differences usually depend on the variation in width of the nacreous layer. The outer wall varies less in thickness. Sieve elements without the nacreous layer may have rather thick walls, but such walls have not been observed to reach the diameter of those characterized by a nacreous thickening.

The nacreous thickening varies from a thin layer, sometimes hardly distinguishable from the outer wall (Fig. 13), to one reaching almost to the center of the cell (Fig. 11). The inner limit of the nacreous thickening is uneven, although this is not always evident in cross-sections. The unevenness results in part from the absence of nacreous thickening where sieve areas occur; in thick nacreous walls the sieve areas appear as deep depressions (Figs. 3, 4, 8, at *s*). In some species, additional, less pronounced waviness of the inner wall occurs independently of the sieve areas (Fig. 7).

The nacreous wall in the secondary phloem considered in this study did not show any special affinity for various dyes; in fact, it was found to be difficult sometimes to make this wall clearly visible in stained preparations. In contrast, the primary phloem often shows deep staining of the thick walls of young sieve elements.[4] Standard microchemical tests revealed no unusual characteristics of nacreous walls. They showed a positive reaction in tests for cellulose; they reacted less strongly than the outer walls in tests for pectic materials; and, like the outer walls, they showed no indication of lignification. Earlier investigators considered the nacreous wall to be highly hydrated,[4] but the present study yielded no evidence for such hydration (see also Cheadle and Esau[3]).

The wall thickening of primary sieve elements usually becomes reduced when the cell ages. The nacreous wall in the secondary phloem shows variable behavior. In some species the wall shrinks; in others it remains thick in the non-conducting phloem. *Liriodendron tulipifera* exemplifies shrinkage of the inner wall. Functional sieve elements of this species appear in Figure 8. A somewhat older cell with partially shrunken wall is shown in Figure 9. During the crushing of old elements the nacreous wall can hardly be discerned from the outer wall (Fig. 10). The cause of this shrinkage has not been determined. In the primary phloem it has been interpreted as evidence of dehydration,[4] but the matter needs further study. *Talauma* sp. may be used to illustrate retention of thick nacreous walls. In the partially crushed sieve elements of this species in Figure 12 the nacreous wall (*n*) is clearly evident. The appearance of younger sieve elements of the same species, taken from near the cambium, is shown in Figure 11.

The development of the nacreous thickening was not considered in the present study. Nevertheless, one may state that the thickening is characteristic of mature

elements, that is, elements without nuclei and with differentiated sieve areas, located next to the vascular cambium and also in older parts of the phloem. In some collections, cells with nacreous thickening at maximum development (Fig. 6, *n*) were flanked on the cambial side by immature elements completely devoid of inner thickenings (Fig. 6, sieve elements with companion cells, *c*). In longitudinal sections such immature cells were found to contain nuclei. These observations suggest that the development of the nacreous wall is associated with the differentiation of sieve elements to a stage usually associated with the functional state.

Eventually the nacreous thickening will have to be evaluated with reference to the wall classification established by the wood anatomists.[5] The classification of the nacreous wall as primary or secondary is part of a general problem of classification of wall layers that requires re-examination in view of the information on wall structure obtained by the use of the electron microscope.

An important part of the present study was concerned with the frequency and distribution of the nacreous wall among the dicotyledons.Comparison of 240 individual plants of 142 species in 121 genera suggests the following grouping of the dicotyledons with regard to wall structure. In this grouping the total wall thickness refers to the wall extending from the middle lamella to the lumen.

A. *Nacreous wall not revealed by light microscopy.* (Total wall thickness ± 1 μ.) Examples. Species examined in *Aesculus, Alnus, Carpinus, Carya, Castanea, Casuarina, Catalpa, Celtis, Cephalanthus, Cornus, Crataegus, Drimys, Fagus, Fraxinus, Gleditsia, Grevillea, Gymnocladus, Hydrangea, Juglans* (Fig. 1), *Koelreuteria, Liquidambar, Morus, Passiflora, Paulownia, Populus* (Figs. 2, 5), *Ptelea, Robinia, Salix, Syringa, Tilia, Ulmus, Wisteria.*

B. *Nacreous wall relatively thin, presence sometimes questionable.* (Total wall thickness from below 1 μ to about 3 μ.) Examples. Species examined in *Buxus, Kalmia* (Fig. 13), *Prunus, Quercus, Rhododendron, Spiraea, Vaccinium.*

C. *Nacreous wall moderately to very thick, typically conspicuous.* (Total wall thickness from about 3 μ to almost half of cell diameter.) Examples. Species examined in *Annona, Asimina, Calycanthus* (Cheadle and Esau[3]), *Cananga, Chimonanthus* (Cheadle and Esau[3]), *Cinnamomum, Cryptocarya, Exochorda, Laurelia* (Fig. 7), *Laurus, Lindera, Liriodendron* (Fig. 8), *Magnolia* (Figs. 3, 4, 6), *Michelia, Paeonia, Persea, Peumus, Phoebe, Rhamnus, Talauma* (Fig. 11), *Umbellularia.*

Some information on the consistency of the occurrence and amount of nacreous thickening within families was obtained for those in which several genera were examined. In some families all representatives investigated had no nacreous walls. For example, no nacreous layer was observed in 4 species of 3 genera of Berberidaceae; in 3 of 2 of Bigoniaceae; in 4 of 2 of Juglandaceae; in 5 of 5 of Lardizabalaceae; in 3 of 3 of Oleaceae; in 3 of 2 of Winteraceae. In some other families all representatives had nacreous walls. For example, such walls occurred in 5 species of 4 genera of Annonaceae; in 3 of 2 of Calycanthaceae; in 7 of 4 of Magnoliaceae. A third group of families contained species with and without nacreous walls. In Lauraceae, for example, 14 species of 10 genera had a conspicuous, often thick, nacreous wall, but 1 species of 1 genus (*Sassafras*) was obviously devoid of this layer. In the Rosaceae 3 species in 3 genera (*Exochorda, Prunus, Spiraea*) had more or less thick inner walls; 5 species in 5 genera (*Amelanchier, Chaenomeles, Crataegus, Pyrus communis* L., *Sorbus*) did not have such walls; and 1 species in 1

genus (*Pyrus Malus* L.) had a small amount of thickening in some sieve elements and not in others.

Different species of the same genus may vary. The example in *Pyrus* was given in the preceding paragraph. Further, *Quercus alba* L. appeared to be free of nacreous thickening, but *Q. coccinea* Muenchh. and *Q. rubra* L. had thin inner layers. Sometimes one of two collections of the same species (e.g., *Betula lutea* Michx.) showed indications of thin nacreous walls, the other did not. When nacreous walls were definitely present, collections made during both dormancy and growth had such walls.

The occurrence of nacreous thickening was examined in relation to two areal measurements of the sieve elements: transectional area of the cell, obtained by multiplying the radial and tangential diameters, and transectional area of the lumen, expressed as per cent of the transectional area of the sieve element (termed "lumen per cent" in the following). In the calculations, each plant was treated separately because of the frequent discrepancies between different collections of the same species with regard to width of sieve elements and sometimes to thickness of nacreous walls. The discrepancies may be accounted for in part by differing vigor of growth of the plants. Of the 240 plants, 71 in 45 species were classified as having nacreous walls, 169 in 97 species as lacking such walls. Two genera, *Azara* and *Quercus*, were represented in both groups, and the doubtful cases were placed in the group without nacreous walls. In some species representatives of different collections fell into different size classes. Although the walls devoid of nacreous thickenings varied somewhat in thickness, they were all treated as being 1 μ wide.

TABLE 1

PER CENT OF PLANTS IN SIZE CLASSES OF TRANSECTIONAL AREA OF SIEVE ELEMENTS

GROUP OF PLANTS	SIZE CLASSES OF AREAS (μ^2)						
	Up to 101	101–300	301–500	501–700	701–900	901–Above	1000
Without nacreous layer: 97 species, 169 plants	1.8	27.2	26.0	16.6	14.2	14.2	
With nacreous layer: 45 species 71 plants	4.2	22.6	46.5	9.8	9.8	7.1	

Table 1 shows the distribution of plants, represented as the per cent of the total number of plants in each group, in the various classes of sizes of transectional areas of sieve elements expressed in μ^2. According to this table, species with nacreous walls show a greater concentration of plants in the smaller size classes than do species without such walls. Over 73 per cent of plants with nacreous walls fall within and below the 301–500 class, whereas only 55 per cent of plants without these walls fall into the same grouping of classes.

Table 2 gives for each of the two groups (with and without nacreous layers) the per cent of the total number of plants that occurred in each lumen per cent class. According to this table, the species without nacreous walls are concentrated in the high per cent classes, whereas the species with the nacreous layer are scattered through 9 of the 10 classes but predominate in the low per cent classes. The scattering is due mainly to the variability in thickness of the inner layer. The other factor that effects variations in lumen per cent is the transectional dimension of cells. At the same wall thickness a smaller cell obviously has a lower lumen per

cent than a larger cell. The use of one average figure for wall thickness in species without nacreous walls may have unduly reduced the per cent lumen in the narrower sieve elements of this group.

TABLE 2

PER CENT OF PLANTS IN SIZE CLASSES OF TRANSECTIONAL AREA OF LUMEN EXPRESSED AS PER CENT OF TRANSECTIONAL AREA OF SIEVE ELEMENT

GROUP OF PLANTS	LUMEN PER CENT CLASSES									
	Up to 11	11–20	21–30	31–40	41–50	51–60	61–70	71–80	81–90	Over 90
Without nacreous layer: 97 species, 169 plants	...	...	...	...	...	...	8.9	40.8	47.9	2.4
With nacreous layer: 45 species, 71 plants	15.5	16.9	26.8	14.1	7.1	7.0	7.0	4.2	1.4	...

The calculations show that, in a high percentage of species with a nacreous wall layer, this layer materially reduces the transectional area of the lumen in sieve elements (Table 2). The increase in wall thickness brought about by the presence of the inner layer is not accompanied by an increase in transectional dimensions of sieve elements; on the contrary, the group of 45 species with nacreous walls had a higher proportion of representatives with relatively narrow sieve elements than did the group of 97 species without the nacreous walls (Table 1). Furthermore, the widest sieve elements, with transectional areas of about 1,000 μ^2, occurred in the group without the inner layer (e.g., species of *Ailanthus*, *Celastrus*, *Juglans* [Fig. 1], *Passiflora*, *Paulownia*, *Ulmus*). The narrowest elements, with transectional areas less than 100 μ^2, however, were found in both groups (e.g., species in *Buxus* and *Calluna* with nacreous walls; *Casuarina* and *Hypericum* without nacreous walls).

As was mentioned at the beginning of this paper, the presence of thick walls in sieve elements may be placed alongside the developmental reduction of potential length of these elements as a feature that would seem to impede the flow of solution through the conduits. It is conceivable that sieve plates constitute highly permeable parts of the wall and that addition of these structures does not materially impede the movement through the conduit, even if this movement is visualized as a mass flow. In this connection, the absence of nacreous thickening on sieve areas (Figs. 3, 4, 8, at *s*) strengthens the commonly held view that the sieve areas have an important role in the movement of materials from sieve element to sieve element. The narrowing of the lumen by deposition of nacreous wall layers is more difficult to relate to a mass-flow mechanism, even if this wall is highly permeable.

Moderately thick, markedly lobed walls and sieve plates might offer less obstruction to a diffusional type of movement, particularly one involving transport of solution along interfaces. Both the sieve plates and the unevenness of moderately thickened walls might be interpreted as increasing the interface area of the cytoplasm. However, as is well known, various other assumptions have to be made if the rapid transport of materials is interpreted as a diffusional movement.

Our understanding of the function and structure of the phloem is still inadequate to permit us to adopt one or another concept of translocation to the exclusion of others.[1] The present paper adds yet another structural aspect that must be taken into account in formulating hypotheses of food movement in the phloem tissue. Current concepts of translocation do not include the consideration of mechanics of

flow of materials through the frequently occurring sieve elements with extremely thick walls, especially those whose lumen is almost occluded.

Summary.—Sieve elements that have reached the stage of development commonly assumed to be mature and functional show a wide range of variation in thickness of walls. Some species have more or less thin homogeneous walls, others have a distinct inner thickening—the so-called nacreous thickening—which varies from a barely perceptible layer to one that almost occludes the lumen of the cell. In the present study 45 species of a total of 142 species of dicotyledons had the nacreous layer. Thus the thickening is not an unusual feature and must be taken into account in formulating concepts of translocation.

We wish to acknowledge the assistance of Mr. Charles H. Lamoureux, who made the initial survey of the species for the presence of nacreous walls and carried out the measurements.

[1] K. Esau, H. B. Currier, and V. I. Cheadle, *Ann. Rev. Plant Physiol.*, **8**, 349, 1957.
[2] K. Esau and V. I. Cheadle, *Acta Bot. Neerl.*, **4**, 348, 1955.
[3] V. I. Cheadle and K. Esau, *Univ. Calif. Publ. in Botany*, **29**, 397, 1958.
[4] K. Esau, *Bot. Rev.*, **5**, 373, 1939.
[5] Committee on Nomenclature, International Association of Wood Anatomists, *Trop. Woods*, No. 36, p. 1, 1933.

INFLUENCE OF THE NUCLEOLUS ON MITOSIS AS REVEALED BY ULTRAVIOLET MICROBEAM IRRADIATION

BY MARY ESTHER GAULDEN AND ROBERT P. PERRY*

BIOLOGY DIVISION, OAK RIDGE NATIONAL LABORATORY,† OAK RIDGE, TENNESSEE

Communicated by Alexander Hollaender, April 22, 1958

Nucleoli are found in most plant and animal nuclei. Their morphological characteristics and cyclic changes during mitosis have been studied extensively. Although evidence is accumulating that indicates a relatively high turnover rate of ribonucleic acid (RNA) and protein in the nucleolus,[1–3] the specific role of this structure in the cell's activity is still in the realm of speculation.

Incidental observations by several workers have suggested a relation between nucleoli and mitotic activity of cells. McClintock[4] noted in maize that microspores with abnormal amounts and distributions of the nucleolar organizing body showed slower rates of nuclear division than did spores with unaltered chromosomes; unfortunately, the nucleolar effects were complicated by changes in number and sequence of genes. Philp and Huskins[5] and McLeish[6] observed that absence of nucleolar organizers in microspores and root tip cells is accompanied by lack of division of these cells. La Cour,[7] McLeish,[8] and Darlington and Haque[9] found that micronuclei in plant cells survive and undergo mitotic changes only if they contain one or more nucleolar organizers.

An excellent tool for determining whether a relation does in fact exist between the nucleolus and mitotic activity is the ultraviolet microbeam as developed by Uretz

and colleagues[10–13] for selective irradiation of cell structures. In the present study the ultraviolet microbeam was used to irradiate the nucleolus and non-nucleolar portions of the nucleus of the grasshopper neuroblast in vitro. The cells were then individually followed by microscopic observation to determine their subsequent mitotic activity. It was found that exposure of only one nucleolus to ultraviolet radiation at stages from late telophase to the middle of middle prophase usually results in immediate and permanent cessation of mitosis. This observation strongly suggests that the nucleolus is directly involved in the mitotic process.

MATERIALS AND METHODS

The neuroblast of the embryo of the grasshopper *Chortophaga viridifasciata* (De Geer) is excellent material for the present study. It is a large cell, about 25 μ in diameter. The various stages of mitosis are highly resolvable and can be readily identified in the living neuroblast. The cell can be separated from the embryo and cultured so as to provide the good optical specimen required for the ultraviolet microbeam apparatus. In the mature living condition, the neuroblast contains two refractile, highly irregularly shaped nucleoli approximately 3 μ in diameter. The nucleoli assume this form in late telophase and keep it until late prophase, when they become smoother in outline and disappear in very late prophase. The nucleoli are formed at a subterminal position on one of the longest pairs of chromosomes and consequently lie in the apolar region of the nucleus. They have never been observed to come in contact with the nuclear membrane or to fuse, even though occasionally they are quite close to each other.

Separation of neuroblasts from 13- and 14-day-old embryos was effected by the trypsin-hyaluronidase technique of St. Amand and Tipton[14] and Roberts[15] with a few minor exceptions. Since Shaw's culture medium[16] promotes good growth of neuroblasts for several days, it was used for making the culture preparations. After 30 minutes in the enzyme solution, the embryos were rinsed with three changes (3 minutes each) of Shaw's medium minus calcium and transferred to complete Shaw's medium for dissociation with a pipette. Culture preparations were then made by spreading thinly on a fused quartz cover glass (0.18–0.20 mm. thick) a small drop of the suspension of cells and tissue fragments. The cover glass was sealed over a flat-bottomed depression slide with mineral oil and fixed in position with melted paraffin. The cultures, even those observed for several weeks, were never opened.

The microbeam apparatus has been described in detail by Uretz and Perry.[12] Use is made of a reflecting objective (0.56 N.A.) that allows one to view the specimen and simultaneously to focus onto a particular part of the cell a demagnified image of a pinhole on which there is a real image of an ultraviolet source. The source consists of a General Electric AH-6 lamp and the filter of an aqueous solution of nickel sulfate and cobalt sulfate.[17] The energy flux of biologically effective radiation per unit area was approximately 0.01 erg μ^{-2} sec $^{-1}$ at the focal point of the microbeam. The beam at this point was a little less than 3 μ in diameter. No effort was made to determine the possible influence of visible light after ultraviolet irradiation. Uniform irradiation and observation procedures were, however, followed for all cells.

Cells were irradiated and studied at room temperature (24° ± 2° C.), observa-

tions being made with either bright-field illumination or phase-contrast microscopy. A few cells were observed at 32° C., but this temperature and higher ones proved to be detrimental to the cultures. Since, in general, completely isolated cells ceased dividing several hours after being separated from the embryo, only neuroblasts in a tissue fragment (one cell thick)[18] were used.

Three cells fairly close to one another and in the same stage of division were selected: in one cell one nucleolus was irradiated; in another cell a non-nucleolar portion of the nucleus was irradiated; and the third cell served as a control. In cells selected for irradiation, both nucleoli were visible and were lying more or less at opposite sides of the nucleus (the usual condition). A cell was usually exposed to the ultraviolet microbeam for 3 seconds. A few cells, especially those in very late prophase, were exposed for 4, 6, 12, 30, or 60 seconds. Irradiated cells were observed until they reached prometaphase[19] or metaphase, or for at least 24 hours; observations on some cells that failed to divide extended for as long as 3 or 4 days.[20] It was not always possible to follow specific cells for longer than 24 hours because of increase in number of cells and their movement in the cultures. Nucleolar-irradiated cells that divided were not followed into the subsequent mitosis. If the unirradiated cell failed to divide, which was a relatively rare event, data on the irradiated cells were discarded.

Data were collected on 81 cells irradiated mainly in the middle, late, and very late prophase stages, designated "MP," "LP," and "VLP," respectively. These stages represent a period during which the nucleolus successively is in mature form, undergoes change before disappearing, and disappears (just after beginning of VLP). A few cells were irradiated in stages from late telophase through early prophase; they are herein designated as "premiddle prophase cells" (pre-MP).

On the basis of chromosome diameter, middle prophase can be subdivided into three parts, MPa, -b, and -c; and late prophase into two parts, LPa and LPb. The nucleus of a living neuroblast at the beginning of MP is uniformly filled with thin chromosome threads. By the beginning of LP, the chromosomes are much thicker, and the two chromatids of each are visible; the cross-sections of about seven chromosomes can be counted at one-fourth the circumference of an optical section of the nucleus. At VLP, the chromosomes are considerably shortened and are about the length of prometaphase chromosomes.

OBSERVATIONS AND CONCLUSIONS

"Permanent" Mitotic Inhibition.—Ultraviolet microbeam irradiation of one nucleolus for 3 seconds is sufficient to prevent further division of practically all the neuroblasts irradiated in stages from late telophase through MPa and of about half the cells irradiated in MPb and MPc (Table 1). Since non-nucleolar irradiation of any mitotic stage usually does not prevent division (Table 1), failure of nucleolar-irradiated cells to divide can be attributed to an effect on the nucleolus even though less intense parts of the beam are obviously passing through some of the nucleus and cytoplasm. Irradiation of one or both nucleoli in LP and VLP cells does not usually prevent division of the cell.

These data can best be interpreted to mean that (1) in stages from late telophase through MPa, the nucleolus is directly involved in the mitotic process—this period represents about 58 per cent of the total mitotic cycle, including interphase; (2)

there is a transition period during MPb and MPc in which the nucleolus is losing its ability to influence mitosis—thus some of the cells irradiated in this period divide and some do not; and (3) by LP, the nucleolus is no longer functioning with respect to the mitotic process.

The nucleolar-irradiated cells that fail to divide do not "die" immediately.[21] In some experiments, cells were observed for as long as 3 or 4 days, during which time the non-nucleolar-irradiated and unirradiated cells continued dividing, whereas the nucleolar-irradiated cells underwent very little change, with the occasional exception of a slight chromosome reversion (to be described).

Six cells irradiated in a non-nucleolar region at stages pre-MP and MP did not divide (Table 1). Failure of division may possibly have been caused by proximity of the microspot to the nucleolus. In four of the cells, the exact position of the microspot with respect to the nucleolus unfortunately was not recorded. In two of

TABLE 1

EFFECT OF NUCLEOLAR AND NON-NUCLEOLAR ULTRAVIOLET MICROBEAM IRRADIATION ON ABILITY OF NEUROBLASTS TO DIVIDE

MITOTIC STAGE AT IRRADIATION	NUCLEAR PART IRRADIATED* Nucleolus +	Nucleolus −	Non-nucleolus +	Non-nucleolus −	TOTAL CELLS
Premiddle prophase, pre-MP	0	4	2	1	7
Middle prophase MPa	1	5	5	1	12
Middle prophase MPb	3	3	5	3	14
Middle prophase MPc	3	4	4	1	12
Late prophase LPa	5	1	6	0	12
Late prophase LPb	5	0	7	0	12
Very late prophase, VLP	1	0	11	0	12
Total cells irradiated					81

* Cells exposed for 3 seconds except for several very late prophase cells (see text). Plus sign indicates cells that divided; minus sign indicates cells that did not divide.

TABLE 2

EFFECT OF ULTRAVIOLET MICROBEAM IRRADIATION ON MITOTIC RATE OF MIDDLE AND LATE PROPHASE CELLS*

MITOTIC STAGES	AVERAGE TIME, WITH STANDARD ERROR, TO REACH MID-MITOSIS (24° ± 2° C.) Unirradiated	Irradiated: Nucleolus	Irradiated: Non-nucleolus
Middle prophase	3.1 ± 0.6 hr. (16)	18.0 ± 1.4 hr. (4)	16.2 ± 0.7 hr.† (11)
Late prophase	34 ± 3 min. (13)	38 ± 6 min. (7)	50 ± 5 min. (6)

* Numbers of cells in parentheses. Data taken only from cells observed to reach mid-mitosis or from those for which this time could be fairly accurately estimated.
† Two cells not included in this calculation reached mid-mitosis 30 and 48 hours after irradiation.

the cells (MPb), however, it is definitely known that the microbeam was focused in such a way that the edge of the microspot was about 3 μ from one nucleolus. This observation suggests the diffusion of a radiation-produced substance from the irradiated region to the nucleolus, or the presence of a nucleolar sensitive region that extends beyond the visible boundaries of the nucleolus itself, or scattering of radiation from the microbeam. Further experiments are needed to resolve this point.

Temporary Mitotic Inhibition.—Cells that were nucleolar-irradiated during their transition period of sensitivity (MPb and MPc) and that divided were greatly retarded in reaching mid-mitosis (nuclear membrane breakdown), as were MP cells irradiated in non-nucleolar regions (Table 2). Late prophase cells nucleolar-irra-

diated showed no mitotic delay, but those irradiated in non-nucleolar regions did exhibit some delay that statistical analysis[22] suggests is a real retardation. Cells nucleolar- or non-nucleolar-irradiated at very late prophase for as long as 30–60 seconds show little or no mitotic delay. These data indicate that the cell becomes less sensitive to ultraviolet microbeam irradiation as it approaches mid-mitosis and that this loss of sensitivity is more pronounced in nucleolar- than in non-nucleolar-irradiated cells.

Morphological Changes.—The only morphological change observed in the nucleolus after irradiation was an occasional slight decrease in size. Irradiated nucleoli disappear in the usual manner if the cells reach very late prophase.[23] The fragmentation and spheration of nucleoli observed by Carlson and McMaster[24] after whole-cell exposure of neuroblasts to monochromatic ultraviolet radiation were not observed after microbeam irradiation. The reason for this lack of spheration, even in a few cells irradiated with a 30-μ microspot, remains to be determined. It seems plausible, however, to consider the nucleolus as having been "inactivated" structurally and functionally by the microspot of ultraviolet. The dose of polychromatic radiation in the microspot focused on the nucleolus in a 3-second exposure is 3×10^6 ergs cm^{-2}, about 1,500 times the mean effective dose of 2650 A found by Carlson and McMaster to be the most effective in producing spheration. This tremendous quantity of radiation most probably alters the nucleolar material so drastically, perhaps by denaturing it, that spheration is not possible.

Mitotic retardation after non-nucleolar irradiation of MP and LP cells is associated with a change in the chromatin best described as "reversion"—the chromatin assumes an appearance resembling that of chromatin of earlier mitotic stages. This phenomenon also occurs occasionally in nucleolar-irradiated cells, but it is slower and considerably less extensive than that observed in non-nucleolar-irradiated cells. Reversion has been observed in neuroblasts of embryos exposed to X-radiation[25, 26] or to ultraviolet radiation.[27] The present study reveals that reversion of chromatin occurs eventually throughout the entire nucleus (15–20 μ in diameter) although irradiation is confined to an area less than 3μ in diameter.

Immediately after non-nucleolar irradiation, the chromatin at the focal point of the microbeam "pales," as described by Uretz *et al.*[10] and Perry[28] to give a clear region, the size of the microspot, in the nucleus. The remainder of the chromatin continues prophase development for about 15–20 minutes, after which the chromatin immediately around the paled spot begins to show reversion. Within the next 5 minutes, all of the chromatin in the nucleus has undergone reversion, the greatest extent of which is to the early prophase condition. If a microspot of chromatin is paled at one side of a nucleus, a longer time is required for reversion of all the chromatin, i.e., about 10–15 minutes. The chromatin reappears in the microspot about 1 hour after irradiation; several hours later, the cell begins to recover and progresses through prophase again.

DISCUSSION

Although the present study does not indicate the specific role of the nucleolus, it does demonstrate that this structure is directly involved in the mitotic process. This makes more plausible the speculation of Norman[29] to the effect that the nucleolus, or possibly the nucleolar organizer, is the cell structure inactivated to give the

non-genetic effects of ultraviolet radiation observed in *Neurospora* conidia. The effect of the ultraviolet microspot on the neuroblast nucleolus is assumed to result from direct damage to the nucleolus; there is no evidence that implicates the nucleolar organizer.

Since it has been shown[30, 31] that deoxyribonucleic acid (DNA) synthesis does not occur after very early prophase in the neuroblast, the cessation of mitosis that results from irradiation of a nucleolus at early and middle prophase cannot be related to disturbances in DNA synthesis. Removal of nutrient yolk from cultures of grasshopper embryos has been shown to affect the ability of neuroblasts in stages through MP and LP to complete mitosis but to have no effect on the mitotic progress of VLP cells.[32] Evidently, the neuroblast requires extracellular materials for completion of the major part of the prophase processes. That the nucleolus may be involved in the utilization by the cell of such materials is suggested by autoradiographs of neuroblasts cultured with tritiated cytidine.[33] These indicate that the nucleolus can incorporate cytidine (into its RNA, presumably) at mitotic stages from late telophase through MPb.[34] The nucleolus appears, therefore, to be metabolically active in those mitotic stages in which it is susceptible to ultraviolet radiation.

Vincent[35] in his comprehensive review has discussed the many functions that have been ascribed to the nucleolus. The most attractive is that proposed by Caspersson[36, 37]: the nucleolus is connected with protein synthesis and the production of cytoplasmic RNA. Evidence to substantiate this view is equivocal, but data obtained with a number of techniques on a variety of cells are accumulating[1, 3, 35, 38–40] that point to the relation of the nucleus to formation of at least part of the cytoplasmic RNA and protein. That the synthetic capabilities of the nucleolus are requisites for the mitotic process can be inferred from the present work. Further studies utilizing a monochromatic ultraviolet microbeam and labeled RNA precursors may reveal the importance of nucleolar RNA to mitosis.

SUMMARY

Exposure of one nucleolus in a grasshopper neuroblast to a microspot of high-intensity ultraviolet radiation for 3 seconds permanently stops mitosis of cells irradiated in stages from late telophase to the middle of middle prophase. During the latter half of middle prophase, the cell becomes mitotically less sensitive to nucleolar irradiation and by late prophase is completely insensitive to it. Microspot irradiation of a non-nucleolar region of the nucleus causes mitotic delay but does not usually prevent cell division. These observations support the hypothesis that the nucleolus has an intimate functional relation to the mitotic process.

* Present address: The Johnson Foundation for Medical Physics, University of Pennsylvania, Philadelphia 4, Pennsylvania.

† Operated by Union Carbide Corporation for the U.S. Atomic Energy Commission.

[1] J. Brachet, *Biochemical Cytology* (New York: Academic Press, Inc., 1957), pp. 127–135.

[2] W. S. Vincent, *Science*, **126**, 306–307, 1957.

[3] W. S. Vincent, in *The Beginnings of Embryonic Development*, ed. A. Tyler, R. C. von Borstel, and C. B. Metz (Washington, D. C.: AAAS, 1957), pp. 1–22.

[4] B. McClintock, *Z. Zellforsch. u. microskop. Anat.*, **21**, 294–328, 1934.

[5] J. Philp and C. L. Huskins, *J. Genet.*, **24**, 359–404, 1931.

[6] J. McLeish, *Nature*, **175**, 890–891, 1955.

[7] L. F. La Cour, *Heredity*, Suppl., **6**, 163–179, 1953.

[8] J. McLeish, *Heredity*, **8**, 385–407, 1954.

[9] C. D. Darlington and A. Haque, *Heredity*, **9**, 117–127, 1955.

[10] R. B. Uretz, W. Bloom and R. E. Zirkle, *Science*, **120**, 197–199, 1954.

[11] R. E. Zirkle, in *Advances in Biological and Medical Physics*, ed. J. H. Lawrence and C. A. Tobias (New York: Academic Press, Inc., 1957), pp. 103–146.

[12] R. B. Uretz and R. P. Perry, *Rev. Sci. Instr.*, **28**, 861–866, 1957.

[13] R. E. Zirkle, W. Bloom, and R. B. Uretz, *Proceedings of the International Conference on the Peaceful Uses of Atomic Energy*, **11** (New York: United Nations, 1956), 273–282.

[14] G. S. St. Amand and S. R. Tipton, *Science*, **119**, 93–94, 1954.

[15] H. S. Roberts, *J. Exptl. Zoöl.*, **130**, 83–105, 1955.

[16] E. I. Shaw, *Exptl. Cell Research*, **11**, 580–586, 1956.

[17] M. Kasha, *J. Opt. Soc. Amer.*, **38**, 929–934, 1948.

[18] Neuroblasts lie in a single layer at the ventral surface of the enbryo. The separation technique often resulted in what appeared to be a stripping of portions of this layer from the embryo to give small fragments.

[19] A cell that reaches prometaphase almost invariably completes mitosis.

[20] A number of cells in tissue fragments have been seen in division in the cultures for as long as 2 or 3 weeks. Differentiation has been observed to begin 3 days after preparation of the cultures.

[21] Neuroblasts are considered "dead" when all Brownian movement of mitochondria in the cytoplasm ceases or when the entire protoplast contracts into a highly refractile, amorphous mass.

[22] The authors are indebted to Dr. A. W. Kimball, of the ORNL Mathematics Panel, for the statistical analysis.

[23] One exception was observed: a nucleolus, irradiated 3 seconds at LPb, persisted into midmitosis and remained attached to the nucleolar chromosome until anaphase, when it moved into the cytoplasm and disappeared.

[24] J. G. Carlson and R. D. McMaster, *Exptl. Cell Research*, **2**, 434–444, 1951.

[25] J. G. Carlson, *J. Morphol.*, **71**, 449–462, 1942.

[26] M. E. Gaulden, M. Nix, and J. Moshman, *J. Cellular Comp. Physiol.*, **41**, 451–470, 1953.

[27] J. G. Carlson and A. Hollaender, *J. Cellular Comp. Physiol.*, **31**, 149–174, 1948.

[28] R. P. Perry, *Exptl. Cell Research*, **12**, 546–559, 1957.

[29] A. Norman, *Exptl. Cell Research*, **2**, 454–473, 1951.

[30] M. E. Gaulden, *Genetics*, **41**, 645, 1956 (abstr.).

[31] M. E. Gaulden, *Proc. First Pan-Am. Cancer Cytol. Cong.* (in press).

[32] M. E. Gaulden and K. L. Kokomoor, *Proc. Soc. Exptl. Biol. Med.*, **90**, 309–314, 1955.

[33] The authors are grateful to Dr. J. Herbert Taylor, Botany Department, Columbia University, for this cytidine.

[34] The incorporated material can be removed from the cells by hydrolysis with HCl (60° C. for 8 minutes).

[35] W. S. Vincent, *Intern. Rev. Cytol.*, **4**, 269–298, 1955.

[36] T. Caspersson and J. Schultz, these PROCEEDINGS, **26**, 507–515, 1940.

[37] T. O. Caspersson, *Cell Growth and Cell Function* (New York: W. W. Norton & Co., 1950).

[38] L. W. Goldstein and W. Plaut, these PROCEEDINGS, **41**, 874–880, 1955.

[39] J. Brachet, in *The Nucleic Acids, Chemistry and Biology*, ed. E. Chargaff and J. N. Davidson (New York: Academic Press, Inc., 1955), 496–519.

[40] H. Stich and W. Plaut, *J. Biophys. Biochem. Cytol.*, **4**, 119–121, 1958.

THE IMMUNE STATUS OF LONG-TERM SURVIVORS OF LETHALLY X-IRRADIATED MICE PROTECTED WITH ISOLOGOUS, HOMOLOGOUS, OR HETEROLOGOUS BONE MARROW

By N. Gengozian, T. Makinodan, C. C. Congdon, and R. D. Owen

BIOLOGY DIVISION, OAK RIDGE NATIONAL LABORATORY,* OAK RIDGE, TENNESSEE

Communicated by Alexander Hollaender, March 20, 1958

Protection of mice from the lethal effects of X-irradiation is obtained by the injection of isologous,[1] homologous, or closely related heterologous (rat) bone marrow shortly after X-ray exposure.[2–4] Transplantation and repopulation of the donor's hematopoietic tissues in irradiated recipients has been demonstrated in experiments in which foreign bone marrow was used.[5–12] Excellent recovery in terms of body weight and long-term survival is afforded lethally irradiated mice by postirradiation injection of isologous bone marrow (IBM). Lethally irradiated mice injected with homologous (HBM) or heterologous rat (RBM) bone marrow, although protected from radiation death, show an incomplete recovery as expressed by a marked secondary loss of body weight and high incidence of mortality occurring 3–6 weeks after treatment. Mice surviving this critical period, or "foreign bone marrow reaction" as it is commonly called, show a gradual return to normal body weight by about the sixtieth to seventieth day after treatment and in gross appearance may compare favorably with the isologously treated mice. The physiologic validity of this comparison was refuted in subsequent immunologic studies that showed a great divergence in the ability of these isologously and heterologously treated mice to produce antibodies to foreign red blood cell antigens (RBC). With 950 r-IBM[13] mice the injection of sheep or rat RBC antigens 30 days after treatment resulted in a normal immune response to sheep RBC but a subnormal response to rat RBC; injection of these antigens 60 days after treatment resulted in a normal response to both types. Thus, with respect to these two antigens, the immune mechanism of 950 r-IBM mice returned to a relatively normal status 60 days after treatment.[14] The response of the 950 r-RBM mice to sheep RBC, however, was considerably below normal when the antigens were injected 30 days after treatment; there was no improvement in response of a second group of mice injected 60 days after treatment. This suggests a definite impairment of the immune mechanism.[15]

To establish the temporary or permanent nature of this lowered immune response among mice treated with foreign bone marrow and consequently to contribute to a better understanding of the mechanism involved, we began a study of the immune status of long-term survivors (200–300 days posttreatment) of lethally irradiated mice treated with isologous, homologous, or heterologous rat bone marrow.

MATERIALS AND METHODS

X-Irradiation Conditions.—(C3H × 101)F_1 or (101 × C3H)F_1 male and female mice 12 weeks old[16] were used as irradiated recipients. The irradiation conditions were: 250 kv. at 15 ma; rate in air ~ 160 r/min; target-skin distance, 60 cm.; inherent filtration, 1.0 mm. of Al and added filtration of 1.0 mm. of Al. The mice were placed in a circular perforated lucite container attached to a revolving turntable, and a single total-body exposure of 900–950 r was given. Since these X-ray

doses are an LD_{100} for the strains used, for convenience all mice will be referred to as receiving 950 r.

Bone Marrow Preparation.—Isologous bone marrow (IBM) was obtained from $(C3H \times 101)F_1$ or $(101 \times C3H)F_1$ mice, homologous bone marrow (HBM) from $(LA)F_1$ mice, and heterologous rat bone marrow (RBM) from Sprague-Dawley rats. Marrow obtained from the tibia and/or femurs of the donors was suspended in Tyrode's solution, adjusted to the desired concentration in 1 ml., and injected intravenously within 4 hours after X-irradiation. Mouse marrow was injected at a dose of $\sim 12 \times 10^6$ nucleated cells and heterologous marrow at a dose of $\sim 140 \times 10^6$ nucleated cells per irradiated host.

Serology.—All long-term survivors used in this study were in excellent condition as indicated by body weight and gross appearance. Immediately before antigen injection, the peripheral blood of the mice treated with foreign bone marrow was tested for circulating donor type RBC, the technique described by Makinodan[6] being used for the 950 r-RBM mice and a dextran-human serum hemagglutination and hemolysin technique (Owen, unpublished data) for the 950 r-HBM mice. At intervals of 200–300 days after irradiation and bone marrow treatment, each mouse received a single intraperitoneal injection of 1 ml. of a 12 per cent suspension of sheep or rat RBC. The limited number of survivors among mice treated with foreign bone marrow made a complete antibody production curve infeasible. Instead, mice were bled 18 days after antigen injection,[14] and the serum was titrated for agglutinating antibodies. We thought that this single-point bleeding would suffice, for the purpose of this study, in establishing the relative immune status of the three different groups of irradiated-marrow treated mice.

The agglutinin titration procedure involved the twofold serial dilution method.[14] The reciprocal of the highest dilution of serum giving a definite macroscopic agglutination was defined as the titer, which was then converted to $\log_2$ titer. The statistical analysis was performed by the "Student" t-test, and the difference in response between any two groups was considered significant if $P < 0.01$.

Autopsy.—Some of the mice used in this study were autopsied after being bled to obtain serum. The tissues were fixed in Zenker-formol solution. The sections were stained with hematoxylin and eosin.

RESULTS

The data are shown in Table 1. The individual titers are tabulated to show the range in agglutinin response in each experimental group. Among the 950 r-IBM mice the immune response to sheep and rat RBC antigens was comparable to that of the normal age group. The 950 r-HBM mice showed a significantly lower response to both sheep and rat RBC than did the 950 r-IBM mice. The response of 950 r-HBM mice to sheep RBC was significantly greater than to rat RBC. A considerable depression of the immune response to sheep RBC was observed in the 950 r-RBM mice, and three mice failed to produce any detectable antibody. The mean sheep agglutinin titer for this group was also significantly lower than that obtained with the 950 r-HBM mice. The $\log_2$ titer of 2.3 for the 950 r-RBM mice is comparable to the 2.6 and 2.9 $\log_2$ titers obtained with antigen injection 30 and 60 days after treatment, respectively, in a previous study with heterolhgously treated mice.[15]

All foreign bone marrow–protected mice used in the present study showed 100 per cent donor-type RBC in the peripheral blood at time of antigen injection. Two additional 950 r-RBM mice that had 100 per cent host (mouse) type RBC in their circulation 200 days after treatment were also injected with sheep RBC. The agglutinin $\log_2$ titer of these two was 7.0, comparable to the normal control group (data not tabulated).

Autopsy examination of 19 mice from the 950 r-IBM group revealed a normal appearance of the hematopoietic and lymphatic systems. Twenty-four mice from the 950 r-HBM series showed marked atrophy of nearly all lymphatic tissues, with

TABLE 1

AGGLUTININ RESPONSE TO SHEEP AND RAT RED BLOOD CELL ANTIGENS IN LETHALLY X-IRRADIATED MICE (950 r) TREATED WITH ISOLOGOUS, HOMOLOGOUS, OR HETEROLOGOUS RAT BONE MARROW

	$\log_2$ TITER* IN MICE TREATED WITH:		
Nonirradiated Untreated (Age Controls)	Isologous (IBM)	Homologous (HBM)	Heterologous (Rat) (RBM)
Mice Injected with 1 Ml. of 1 per cent Sheep RBC 200–300 Days after Treatment			
8	7	5	0
9	6	5	0
8	8	5	3
8	7	4	6
8	7	7	6
8	7	5	0
8	7	5	2
8	8	4	2
7	7	6	3
8		2	3
		5	1
		4	1
Mean titer 8.0	7.1	4.8	2.3
Mice Injected with 1 Ml. of 1 Per Cent Rat RBC 200–300 Days after Treatment			
8	7	1	
8	7	4	
7	8	1	
8	5	2	
8	8	2	
9	7	3	
9	8	4	
8	7	1	
9	7	1	
9	7	4	
		2	
		4	
Mean titer 8.3	7.1	2.4	

* Each value represents the results of a single test on one animal.

fibrous replacement of most of the peripheral lymph nodes. Deep scars were visible through the spleen capsule. One animal showed changes in the cervical lymph nodes resembling those seen in the foreign bone marrow reaction.[17,18] Other lymphatic tissues in this mouse did not show the reaction. Major reactive changes were not observed in the lymphatic tissue of other mice of the 950 r-HBM series. The mice in the 950 r-RBM group were not autopsied.

DISCUSSION

The results of this study demonstrate the subnormal immune status of the long-term survivors of the mice lethally irradiated and protected with foreign bone

marrow and, in conjunction with our previous studies, suggest this impairment to be of a permanent nature, *provided* that the foreign transplant persists in these animals. On the basis of information obtained in our previous studies on irradiated mice injected with heterologous rat bone marrow, we concluded that the "foreign bone marrow reaction" occurring in 950 r-RBM mice was caused by a delayed immunologic response of the irradiated host against the antigenic components of the foreign graft, resulting in a chronic in vivo antigen-antibody reaction and its subsequent disease syndromes.[6, 14, 15, 19, 20–23] The alternative possibility, i.e., that the foreign graft produces antibodies against the antigenic components of the host and thereby leads to this in vivo reaction, has been proposed by others.[24–26] The validity of the host versus graft or graft versus host hypothesis is irrelevant to the present discussion. That there is an in vivo antigen-antibody reaction occurring from the third through the sixth week after treatment in lethally irradiated–foreign marrow treated animals is now generally accepted. The tendency, however, has been to consider that mice having recovered from this critical period, as indicated by a body weight increase and improved general appearance, are now tolerant to the graft, or the graft tolerant to the host. The word "tolerant," however, implies (*a*) a cessation of the in vivo antigen-antibody reaction, (*b*) complete and mutual compatibility of host and graft, and (*c*) a normal immunologic responsiveness of the individual toward antigens *unrelated to host or graft*. Our negative findings on the last point necessitate a re-evaluation of the concept of tolerance in these mice. Two tentative explanations for the subnormal immune mechanism may be as follows: (1) tolerance does exist, and the lowered immune response is merely a quantitative expression of the functional antibody tissue remaining in these mice after recovery from the foreign bone marrow reaction, or (2) the in vivo antigen-antibody reaction, reflecting the incompatibility of host and graft, persists in these animals and consequently affects the immunologic capacity of these mice to respond normally to other antigens.

The preliminary histologic observations on the 950 r-HBM mice would appear to substantiate the first postulate. Thus the marked atrophy of lymphatic tissues and fibrous replacement of most of the peripheral lymph nodes indicates a permanent loss of antibody-forming tissue, and a subnormal response would not be surprising. A quantitative reduction of antibody-forming tissue would, however, not be expected to affect the differential response to sheep and rat RBC observed in the 950 r-HBM mice. The lower response to sheep RBC in the 950 r-RBM mice than in the 950 r-HBM mice would also seem to negate this hypothesis, but lack of histologic observations on the former group prevents definitive comparisons. The absence of typical reactive changes as seen during the "foreign bone marrow reaction" in most of the long-term homologously treated mice would seem to favor a state of tolerance. Additional histologic studies on these long-term survivors of the foreign marrow-treated mice is indicated, and a comprehensive study is in progress.

The second postulate, that a persisting in vivo antigen-antibody reaction exists in these mice and results in a lowered state of immunologic reactivity, is formulated as follows: During the period of secondary loss of body weight and increased deaths characterizing the "foreign bone marrow reaction," the in vivo balance of antigen and antibody is at the optimum proportion necessary to effect this delayed immune reaction. Gradually this balance is shifted, as an excess of antigen (rela-

tive or absolute) offsets the fine equilibrium of antigen and antibody required in vivo to give the delayed type of reaction.[27] The animals recover at this time. The physical manifestations of such an "in vivo antigen excess phenomenon" need not be apparent, either at the gross anatomical level or in histologic examination of the tissues. Such a reaction, however, would place a physiologic stress on the animal's immune mechanism and might result in a subnormal response to unrelated antigens. The difference in response to sheep and rat RBC in the 950 r-HBM mice may be described in terms of the recognition factor postulated by Makinodan *et al.*;[14, 21] i.e., a greater response to the more distantly related antigen (sheep RBC) may occur when the immune mechanism is placed under severe stress. The ability of the 950 r-HBM mice to produce more agglutinins to sheep RBC than the 950 r-RBM mice suggests a quantitative expression of this stress on the antibody-forming mechanism. Thus the genetic relation between host and graft may be reflected in the degree of physiologic stress created by this in vivo reaction. The association of the continued presence of the foreign graft with this effect is evident in the normal immune response to sheep RBC in two 950 r-RBM mice which had sloughed their grafts prior to antigen injection. Additional observations supporting the concept of a persisting in vivo antigen-antibody reaction are (*a*) the changing proportions of donor and host RBC in the peripheral blood of several foreign bone marrow chimeras[28, 29] and (*b*) the complete reversal to mouse-type cells that we and others have noted in a small percentage of the 950 r-RBM mice. Indeed, as indicated by Makinodan,[30] the foregoing observations themselves make it difficult to conceive of a state of "tolerance" in these mice.

The results of this study suggest a re-evaluation of the concept of tolerance as applied to the long-term survivors of the foreign marrow–protected mice. The 1957 review by Owen[31] on immunologic tolerance makes apparent the complexities among the various phenomena concerned with modifications of the host's immune response and accordingly cautions one against equating their mechanisms of reaction. Thus, instead of clasifying and typing the foreign marrow–protected mice as "tolerant" or in a state of immunologic "paralysis,"it seems advisable to maintain a separate identity for these phenomena until further investigations provide tests for definitive relationships.

SUMMARY

The immune status of lethally irradiated mice treated with isologous, homologous, or heterologous rat bone marrow was studied by injecting sheep or rat RBC antigens into the protected mice 200–300 days after treatment. The results showed the foreign bone marrow–protected mice to have a subnormal immune mechanism, provided that the foreign transplant persisted in these animals, whereas the isologously treated mice responded in a normal manner.

Two postulates are considered in the interpretation of this subnormal immune mechanism: (1) the lowered immune response is a quantitative expression of the functional antibody tissue remaining in these mice after recovery from the foreign bone marrow reaction, or (2) an in vivo antigen-antibody reaction persists in these animals and consequently affects their ability to respond normally to other antigens. Emphasis is placed on a re-evaluation of the concept of tolerance as applied to the long-term survivors of the foreign marrow–protected mice.

* Operated by Union Carbide Corporation for the U.S. Atomic Energy Commission.

[1] "Isologous" denotes intrastrain differences within a species; "homologous" denotes interstrain differences within a species; and "heterologous" denotes interspecies differences.

[2] E. Lorenz, D. Uphoff, T. R. Reid, and E. Shelton, *J. Natl. Cancer Inst.*, **12**, 197, 1951.

[3] E. Lorenz, C. C. Congdon, and D. Uphoff, *Radiology*, **58**, 863, 1952.

[4] C. C. Congdon and E. Lorenz, *Am. J. Physiol.*, **176**, 297, 1954.

[5] D. L. Lindsley, T. T. Odell, and F. G. Tausche, *Proc. Soc. Exptl. Biol. Med.*, **90**, 512, 1955.

[6] T. Makinodan, *Proc. Soc. Exptl. Biol. Med.*, **92**, 174, 1956.

[7] P. C. Nowell, L. J. Cole, P. L. Roan, and J. G. Habermeyer, *Cancer Research*, **16**, 258, 1956.

[8] C. E. Ford, J. L. Hamerton, D. W. H. Barnes, and J. F. Loutit, *Nature*, **177**, 452, 1956.

[9] O. Vos, J. A. G. Davids, W. W. H. Weyzen, and D. W. van Bekkum, *Acta Physiol. et Pharmacol. Neerl.*, **4**, 482, 1956.

[10] L. H. Smith, T. Makinodan, and C. C. Congdon, *Cancer Research*, **17**, 367, 1957.

[11] N. Gengozian, I. S. Urso, C. C. Congdon, A. D. Conger, and T. Makinodan, *Proc. Soc. Exptl. Biol. Med.*, **96**, 714, 1957.

[12] R. M. Merwin and C. C. Congdon, *J. Natl. Cancer Inst.*, **19**, 875, 1957.

[13] Mice receiving 950 r and an intravenous injection of isologous, homologous, or heterologous rat bone marrow are designated 950 r-IBM, 950 r-HBM, or 950 r-RBM mice, respectively.

[14] T. Makinodan, N. Gengozian, and C. C. Congdon, *J. Immunol.*, **77**, 250, 1956.

[15] N. Gengozian and T. Makinodan, *J. Immunol.*, **77**, 430, 1956.

[16] The 30-day LD_{50} doses for these strains are 756 and 730 r, respectively.

[17] C. C. Congdon and I. S. Urso, *Am. J. Pathol.*, **33**, 749, 1957.

[18] C. C. Congdon, *Radiation Research*, **7**, 310, 1957.

[19] T. Makinodan, *J. Cellular Comp. Physiol.*, **50**, 327, 1957.

[20] N. Gengozian and T. Makinodan, *Cancer Research*, **17**, 970, 1957.

[21] T. Makinodan, I. C. Shekarchi, and C. C. Congdon, *J. Immunol.*, **79**, 281, 1957.

[22] T. Makinodan and N. G. Anderson, *Blood*, **12**, 984, 1957.

[23] T. Makinodan, N. Gengozian, and J. F. Albright, *Ann. N.Y. Acad. Sci.* (in press).

[24] J. J. Trentin, *Proc. Soc. Exptl. Biol. Med.*, **96**, 139, 1957.

[25] D. E. Uphoff, *J. Natl. Cancer Inst.*, **19**, 123, 1957.

[26] D. W. H. Barnes, C. E. Ford, P. L. T. Ilbery, P. C. Koller, and J. F. Loutit, *J. Cellular Comp. Physiol.*, **50**, 123, 1957.

[27] T. Makinodan, H. R. Wolfe, M. Goodman, and R. Ruth, *J. Immunol.*, **68**, 219, 1952.

[28] D. W. van Bekkum and O. Vos, *J. Cellular Comp. Physiol.*, **50**, 139, 1957.

[29] T. T. Odell, F. G. Tausche, D. L. Lindsley, and R. D. Owen, *Ann. N.Y. Acad. Sci.*, **64**, 811, 1957.

[30] T. Makinodan, *J. Cellular Comp. Physiol.*, **50**, 157, 1957.

[31] R. D. Owen, *Federation Proc.*, **16**, 581, 1957.

GENETIC STUDIES OF IRRADIATED NATURAL POPULATIONS OF DROSOPHILA. II. 1957 TESTS

BY WILSON S. STONE* AND FLORENCE D. WILSON

GENETICS FOUNDATION, DEPARTMENT OF ZOÖLOGY, UNIVERSITY OF TEXAS, AUSTIN, TEXAS

Communicated by J. T. Patterson, April 1, 1958

Introduction.—There has been much discussion of the genetic effects on populations of the immediate radiations and fallout and radioactive wastes from atomic and thermonuclear detonations and atomic reactors. Many careful laboratory studies of the effects of various sorts of radiation on different genetic systems have been made. Estimates of the effects on populations have been made from these tests. This approach seemed inadequate to many investigators, especially in establishing the effects on populations of atomic weapons or large-scale use of reac-

TABLE 1*

RESULTS OF CROSSES INVOLVING MATERIAL COLLECTED IN SUMMER, 1957

	P_1 Females				Fertile Pairs				Egg Development						
											Pattern (Per Cent)				
	No.	Per Cent with Lethal	Pairs Tested	Per cent Sterile	No.	Per Cent with Lethal	Days Eggs Counted	Eggs/day ♀	No.	Per Cent Developed	1–19	20–39	40–59	60–69	80–100
Ponape (P)	31	83.9	154	43.5	66	68.2	127	34.9	4426	68.8	2	4	17	22	21
P (random)			111	41.4	50	22.0	118	31.0	3662	87.8	0	4	5	2	39
Majuro (M)	48	91.7	242	53.7	86	84.8	225	24.1	5415	63.7	1	13	26	33	13
M (random)			125	48.8	57	50.9	148	29.5	4361	74.9	1	2	8	18	28
Bikini (B)	92	95.7	341	27.9	218	78.4	486	30.4	14767	57.7	10	45	56	60	47
B (random)			142	10.6	101	26.7	257	30.1	7728	81.8	1	2	7	17	74
Rongerik (K)	25	88.0	111	54.1	38	76.3	64	26.0	1663	63.6	1	4	11	13	9
Rongelap (A)	44	72.7	178	42.7	79	60.8	193	26.0	5011	64.5	2	6	30	10	31
A (random)			99	33.3	63	30.2	166	24.9	4130	83.6	1	1	6	11	44
M × P			140	62.9	20	55.0	56	15.6	871	70.1	1	4	4	2	9
A × M			158	46.2	71	29.2	214	22.6	4837	81.8	2	2	5	12	51
M × B			121	19.0	88	45.5	266	15.4	4107	79.4	0	2	11	27	48
P × A			132	60.6	33	17.9	81	16.6	1343	83.8	0	0	1	5	27
B × P			165	73.3	28	17.9	65	23.2	1506	86.1	0	0	0	5	23
B × A			147	30.6	93	52.7	215	34.9	7494	73.6	4	6	12	27	44
AM × BP			159	38.9	73	17.8	191	24.2	4622	85.7	1	0	5	7	60
BA × MP			101	70.3	21	19.0	50	24.9	1244	86.8	0	0	0	4	17
MB × PA			94	31.9	54	57.4	164	25.2	4127	73.0	0	6	7	18	23
AM × AM	44	88.6	164	27.4	105	72.4	293	30.1	8725	64.0	1	9	30	36	29
BA × BA	44	100.0	177	19.2	130	83.8	330	31.4	10377	57.0	4	29	42	34	21
MB × MB	57	96.5	213	20.2	147	74.5	342	33.5	11463	64.6	2	16	45	48	38
BP × BP	23	91.3	110	45.5	49	77.6	106	27.5	2908	61.5	0	6	19	13	11
MP × MP	14	85.7	65	41.5	28	67.9	58	28.3	1641	60.1	0	2	13	4	9
PA × PA	32	87.5	123	69.5	74	69.5	151	31.1	4700	68.7	0	7	19	18	30
MB × BA			135	15.5	113	56.6	266	31.8	8456	72.4	1	14	15	34	49
AM × MB			137	13.1	100	34.0	241	35.4	8532	78.7	1	8	8	17	66

* Given at the left are the stocks, their letter symbols, and the crosses. Column 1 gives the number of females which gave one or more pairs of fertile progeny tested by brother × sister matings (col. 5). If one or more of these progeny pairs had a lethal or lethal equivalent genetic complex (scored lethal when less than 80 per cent of the eggs laid by a female which had produced progeny developed), their parent pair was scored as having one or more lethals in column 2, "Per Cent with Lethal." The total number of pairs tested and the per cent sterile are given in columns 3 and 4. The results of tests of fertile pairs (cols. 5 and 6) give the number fertile and the per cent showing one or more lethal equivalents. Columns 7–15 give information on egg development. Column 7 gives the days eggs were counted from these pairs, and column 8 gives the average number of eggs laid per day. Columns 9 and 10 give the number of eggs laid by females known to have been fertilized and the per cent of these eggs that developed into adults. The pattern of egg development in the several crosses is shown in columns 11–15. These columns give the number of females in classes with their egg development ranging from 1 to 19 per cent, etc. Only the last class (80–100 per cent) is regarded as normal development—a view supported by the results given diagrammatically in Figs. 1–4.

tors. Therefore, the United States Atomic Energy Commission approved and assisted us to study some isolated populations of *Drosophila ananassae* in the Marshall Islands, including the Pacific Proving Ground, and at Ponape in the eastern Carolines.

The populations studied are of different sizes and associations. We attempted to characterize these populations in several ways in an earlier publication.[1] *Drosophila ananassae* is found in tropical regions around the world and is known from many of the Pacific islands. Tests showed that this species is well suited to these regions because of its superior tolerance of the warm temperature and superior larval competition for food. These tests were made in competition cages, using various other competitor species, including *D. melanogaster*. This latter species could displace *D. ananassae* at 72° F. but was displaced by *D. ananassae* at 77° F. One of us (W. P. Spencer)[1] showed that the incidence and types of visible mutations detectable on inbreeding were of the same general frequency and types as found in other species of this genus. Seecof[1] showed that the distribution and types of chromosomal rearrangements produced by X-rays were comparable to those produced by X-rays in *D. melanogaster* and that frequency of recovered rearrangements followed a pattern similar to that in *D. melanogaster* and in *D. virilis* through the spermatogenesis cycle. A very few small previously unknown paracentric inversions were found in some of the populations, but no other gross chromosomal abnormality which might be attributed to the effects of radiation was found. The frequency of lethals and lethal complexes was determined for several populations collected in the summers of 1955 and 1956.

One of the populations in the northern Marshalls, Bikini, received direct irradiation and fallout, while Rongelap and Rongerik received only fallout, especially from the thermonuclear explosion of March 1, 1954, and again from the tests in 1956. The types and distributions of radioactive debris from fallout are discussed in an United States Atomic Energy Commission[2] publication in detail. This includes measurements of radiation from different localities in this region found at different intervals after March, 1954, in soil, water, animals, and plants—some of the latter used as food by *D. ananassea.*

Experimental.—Populations were collected in August, 1957, on the islands of Bikini, Rongelap, and Rongerik in the northern Marshalls; at Majuro in the southern Marshalls; and on Ponape in the eastern Carolines. These populations were all sampled in August, 1956. In August, 1955, no sample was obtained from Ponape, and the sample from Rongerik atoll came from the island of Eniwetak rather than Rongerik. Only *D. ananassae* is found in the northern Marshalls, but small numbers of *D. melanogaster* and *D. bryani* are found at Majuro, and several additional species are found in abundance on Ponape.[1] The *Drosophila* collected at the several localities were shipped to Austin by air. Individual *D. ananassae* females were allowed to produce progeny, either from fertilizations already accomplished or, if no offspring were produced in a reasonable period, from matings with individual males from the same collection. These F_1's were tested for fertility and for factors influencing viability by inbreeding brother × si ter, by crossing F_1 from different females from the population on the same island, and by crosses between members of populations from different islands. The next generation was

also tested by inbreeding and crossbreeding, the latter including three-way (one recurrent strain) and four-way (no recurrent strain) crosses.

Certain steps must be kept in mind which are necessary to an analysis of the populations as they occur on the islands. We are limited in that we have been able to test the populations only once each year; however, the samples were taken at the same time each year to insure a minimum difference due to environmental cycles. It is necessary to test the progeny of the original flies to determine their genotype and thus that of the population insofar as this is possible. In practice the material is shipped by air to the laboratory; the tests are begun and carried step by step to

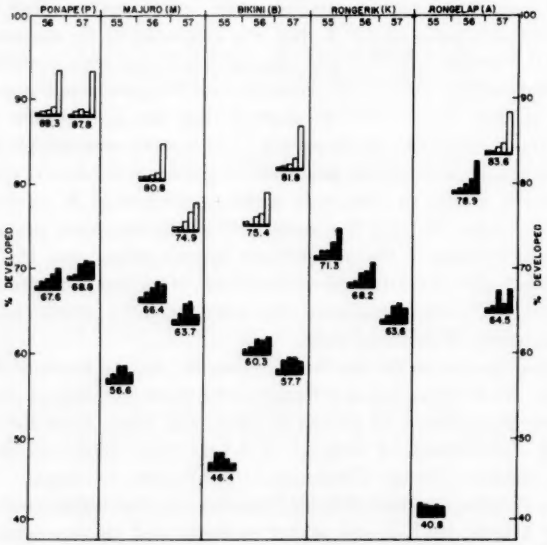


FIG. 1. Egg development within populations. The stippled figures are brother × sister matings; the plain figures are crosses between offspring of different pairs. These were run at the same time with the progeny of pairs, tested both ways when possible. The test years are indicated at the top for each population. The histogram shows the relative percentage of females with egg development of 1–19, 20–39, 40–59, 60–79, and 80–100 per cent from left to right. The average per cent development of all eggs laid is given under each population histogram. The letter symbol for the particular island is indicated with the name.

completion; and then the data are analyzed. This means that, because of lack of original fertile females or because of unpredictable laboratory environmental variation, usually bad food or mold infestation, some tests may be unsatisfactory.

Results.—The data obtained from the tests are given in Table 1. In order to make a more meaningful analysis of this study of populations, comparisons between the tests for 1957 and the two earlier years are shown in Figures 1, 2, and 3. Figure 4 gives some comparable tests of several strains in two other species which had been reported earlier.[3] The information given in the table and figures is explained in detail in each case. Some comparisons with data[1] for 1955 and 1956 are pertinent, in

addition to the results of inbreeding and crossbreeding which will be considered in detail. We will present data for 1955, 1956, and 1957 in that order. The average per cents fertile of the stocks and their initial crosses were 53.0, 58.3, and 56.8 per cent, while those of their F_1 and F_2 progeny were 70.6, 66.4, and 64.3 per cent.

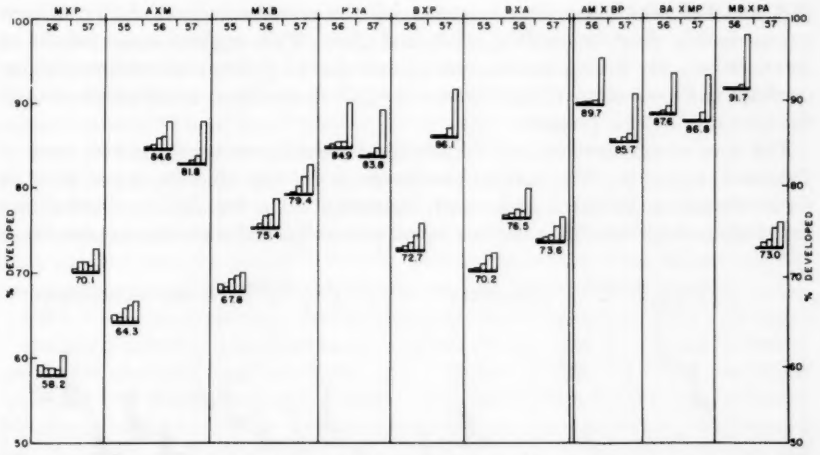


FIG. 2. Six sets of crosses for the two or three years indicated between members of populations from different islands and three double (four-way) crosses between different heterozygotes for maximum heterosis.

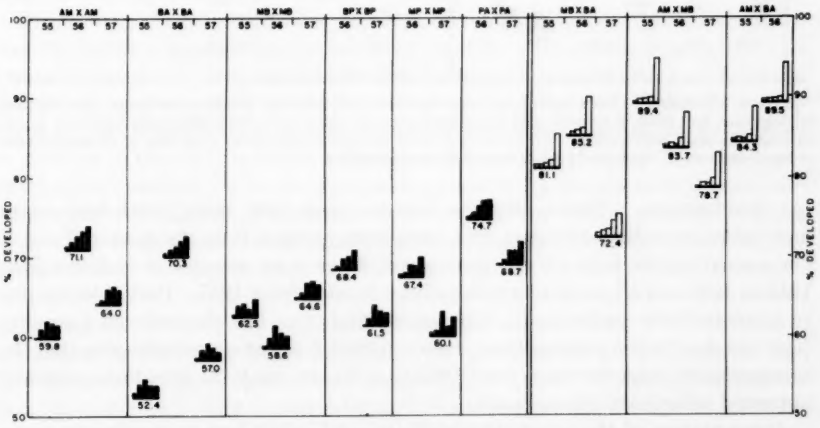


FIG. 3. Six sets of brother × sister matings of heterozygotes from matings between populations from different islands. Three sets of three-way crosses (one recurrent strain).

These averages show a superiority of the heterozygotes, indicating that certain genetic factors adversely influenced fertility in the populations. It is of interest that the Bikini population was ordinarily the most fertile of the four or five populations tested by inbreeding. Although in 1956 it was about the same as Rongelap

and Ponape, it was more fertile in all other individual comparisons. The three-year average fertilities are: Bikini, 70.3 per cent; Ponape, 62.0; Rongelap, 57.6; Majuro, 51.6; and Rongerik, 51.0.

The average number of eggs laid per day also suggests some effect of heterozygosity. The averages for the stocks and their crosses for the three years are 26.2, 24.9, and 25.7, which are very consistent; but the averages for tests of their heterozygous female progeny are 29.7, 19.3, and 29.4. This suggests some benefit of heterosis but only if we suppose some adverse change in environmental conditions (perhaps bad food) occurred in 1956 after the parents had been tested which reduced the fecundity of their progeny.

The data on egg development for the three years are presented in two ways in Figures 1, 2, and 3. The average percentage of the eggs that developed from all fertile females is indicated under each histogram. The females are divided into five classes, which show their relative reproductive efficiency measured as percentage

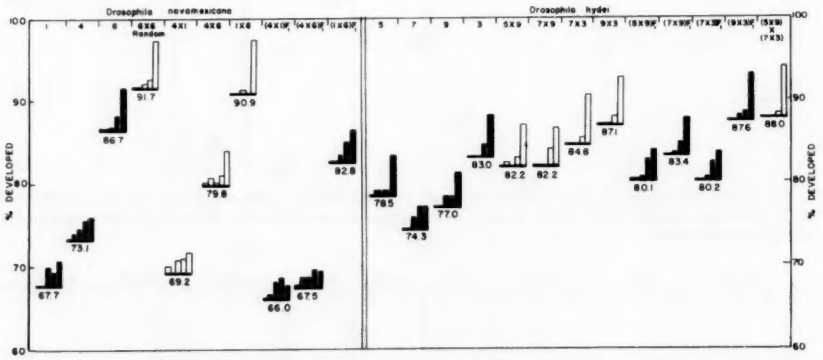


FIG. 4. Comparable tests with two other species with brother × sister matings, the stippled histograms, and random crosses within a population or crosses between individuals from two populations, the plain histograms. The numbers along the top margin refer to stocks of *Drosophila novamexicana* and of *Drosophila hydei* from different localities.

egg development. Almost all these females came from pairs which had one or more lethal or lethal equivalent gene complexes, judging from the data in Table 1. On comparing the tests for the three years, there is an increase in viability from 1955 to 1956 and a less pronounced decrease from 1956 to 1957. Part of this is due to uncontrollable variations in laboratory conditions but the reduced fecundity (eggs per day) of the progeny from crosses in 1956 does not support the idea that the comparatively large increases from 1955 to 1956 are solely the result of a generally improved laboratory environment.

A comparison of the reproductive efficiency of inbreeding by brother × sister matings and random mating between offspring of different females shows the consistent and appreciable advantage of random mating. This averages about 18 per cent better egg development, with Majuro showing the least difference. There are 29 tests with which to make more general comparisons of the effect of inbreeding using brother × sister matings (Figs. 1 and 3). The average egg development for the 29 is 63.3 per cent, with 21 tests falling between 56.6 and 68.8. The five above

this range include the very small population from Eniwetak island (Rongerik atoll) collected in 1955. This population had disappeared by 1956, and the other two samples are from Rongerik island. The other four high tests include all tests involving the Rongelap population collected in 1956. The three tests falling below 56.6 include the very low Bikini and Rongelap populations and their heterozygote in 1955. The random-mated pairs within populations can be compared to the crosses between members of different populations in 1956 and 1957. The average egg development for random matings is 81.8 per cent (range 74.9–88.3), while that of crosses between populations is 77.3 per cent (range 58.2–86.1). With the two crosses between Majuro and Ponape removed, this value is 79.9 per cent. These crosses were not very successful either year, falling into the low classes with A × M, M × B, and B × A for 1955, which involved the irradiated populations of Bikini and Rongelap. The three- and four-way crosses (Fig. 2 and 3) showed the gain from heterosis and from having heterozygous parents. The average egg development was 84.2 per cent (range 72.4–91.7), with all three tests below 80 per cent development occurring in 1957 and involving the MB heterozygotes.

There is another example of the superiority of the heterozygote in the 1955 tests. The heterozygotes between Bikini and Rongelap, Majuro and Bikini, and Rongelap and Bikini when inbred gave a better egg development by about 10 per cent than the averages of their parent stocks inbred. This was not true for the tests for stocks and crosses in 1956 and 1957. In those two years the average for all heterozygotes was almost identical with the averages of their parent stocks—in each case the detrimental factors could become homozygous in the eggs from the brother × sister matings. In 1955 the stocks seemed to carry factors (homozygous), which reduced the effective egg development of their offspring.

Discussion.—The thermonuclear device of March 1, 1954, was exploded seventeen months before our collections were begun in 1955. To show a genetic effect of irradiation on populations under these circumstances, it is necessary to establish the population cycles on both irradiated and control populations. If irradiation injured any of the populations enough to measure, we must establish this as an effect in addition to the other population fluctuations from year to year. It is necessary to establish consistencies in the data because laboratory environmental variations may mask or exaggerate differences. Differences in population structure may influence the decay of variability and the rapidity of natural selection in reducing any excessive burden of genetic abnormalities that might be produced. The *D. ananassae* population on Ponape is large and in competition with a number of other species of *Drosophila*. It has given very similar tests the two years (Fig. 1). The Majuro population is also fairly large, although very much smaller than Ponape. The Bikini population has been small and sparse all three years. The Rongelap population was larger, especially the second year, but was reduced the third year. The exceptionally large and vigorous population on Rongelap the second year occurred on the abundant fruit at the site of the native village. Much of the pandanus and papaya had been cleared away in rebuilding the village for the natives, who returned in June 29, 1957.[2] The amount of fallen fruit was much reduced on that island in August, 1957, partially by the action of the animals brought in by the Marshallese. The population of *Drosophila* on Eniwetak in 1955 was much smaller than on the other islands and had disappeared by 1956. The population on Rongerik

was also very small in 1956 and 1957. Despite the several disadvantages of these populations for study, they have an exceptional advantage because they are isolated populations. No other isolated populations of *Drosophila* have been studied except the very small populations of *D. novamexicana* which occur infrequently along desert streams (Fig. 4). The *D. hydei* populations also presented in Figure 4 are thin and scattered but part of the extensive desert *D. hydei* population.

The three- and four-way tests give a measure of the possible environmentally induced variation. These were very consistent, and in fact all the tests that fell below 80 per cent egg development were in 1957. The maximum reduction below the mean of these tests was 11.8 per cent. The reduction below 100 per cent development is due to the sum of adverse environmental and chance errors and dominant adverse genetic factors. All other tests had the additional genetic hazard of detrimental recessive factors (or the recessive component of their action) either homozygous or becoming homozygous in the tests. Furthermore, the egg-development test with a continuous range from 1 to 100 per cent—zero developing eggs are classed as sterile—allows us to measure the summed effects of the supposedly numerous genes with small effects as well as those with gross effects, such as lethals. The distribution of egg development in histograms shows this as well as we can with the samples we have.

The inbred tests show the following consistencies: taking the lowest control population, Majuro, 1955, as one limit, the range is from 56.6 to 68.8 per cent for 21 of the 29 tests. The mean of all tests is 63.3 per cent. The heavily irradiated Bikini (1955) population was 16.9 per cent below the mean; Rongelap (1955) was 22.5 per cent and their heterozygote 10.9 percent. The three-way crosses that year were high, and the control Majuro population was only 6.7 per cent below the mean of the inbreds. The simplest explanation of the data is that the irradiation of Bikini and Rongelap populations increased the number of detrimental genetic factors in those populations. Rongelap recovered from the major 1954 fallout to about the normal level by 1956 or before, but the Bikini population which received most radiation from the 1954 and 1956 test detonations had the lowest egg development of any population in 1956 and in 1957.

A second consistency is the uniformly high egg development in tests involving the Rongelap population collected in 1956. Four of the five high inbred tests of the 29 for all three years involved the Rongelap 1956 stock; the other one was the Eniwetak population which we could sample only once, as discussed previously. Viewed another way, of the nine inbred tests in 1956, all four involving Rongelap gave above 70 per cent (range 70.3–78.9 per cent) egg development with Rongelap inbred 78.9 per cent, while the other five were below 70 per cent (range 60.3–68.4 per cent). The three- and four-way tests in 1956 were good. We consider that this local population had achieved a high viability with a low level of detrimental genetic factors. It probably contributed to raising the general level on Rongelap Island, although it was locally eliminated by man and his animals.

We have already discussed the general heterosis in egg development on crossbreeding rather than inbreeding here and elsewhere;[1] however, these populations may be compared to those of other species tested in the same way. The *D. novamexicana* populations (Fig. 4) are smaller than any of these *D. ananassae* populations, with the possible exception of those from Rongerik atoll. The *D. hydei* popu-

lations are not so dense anywhere as the Ponape or Majuro populations, but they are scattered widely in the desert region.

These *D. hydei* populations contain fewer detrimental factors and are more uniform inbred or crossbred than the *D. ananassae*, and *D. novamexicana* is more variable than *D. hydei*. Population 6 and its cross to and heterozygote with population 1 is clearly superior to *D. ananassae*. Populations 1 and 4 and the crosses and heterozygotes involving 4 (which carries a dominant factor that reduces egg development from heterozygotes with the other stocks[3]) are in the same general range with *D. ananassae*. Both these other species are somewhat better laboratory species than *D. ananassae*, which is not one of the better laboratory species. Nevertheless, the data show us that quite small populations like those on Rongerik atoll can recover within a year and a half from the one severe fallout. The larger Rongelap population, with perhaps somewhat heavier fallout in 1954, required more time but could recover in 2.5 to 3.5 years. Even the Bikini population, which received direct irradiation as well as the heaviest fallout of a test locality, can survive and recover almost to the general level of these populations, probably aided by its greater fertility, which may reflect the more stringent selection at this locality.

The time scale for recovery should be stated in other terms. The temperature in this region varies around 75°–80° F.; so the generation time of *D. ananassae* is from 15 to 20 days or 18 to 24 generations per year. We can say that the Rongerik populations, with their small size, recovered within about 26 generations and the Rongelap within 40 generations. Bikini was still somewhat low. This would place comparable dates in human history at the time of Columbus (Rongerik) and the Crusades (Rongelap). Further, these *Drosophila* average more than 25 eggs per day per female, and many of the flies we captured were several days old; some few were old and ragged, which might mean several weeks. This could mean 100 or more fertilized eggs laid per pair, a margin of reproductive effectiveness a number of times that possible by man. Another factor is the persistent radiation from fallout. Bikini and even Rongelap were appreciably above the general world background level of radiation in 1955. With the added test in 1956, this was still above average at Bikini and was slightly above the world average level at Rongelap even in 1957.[2] This means that the mutation rate in these areas was raised above the general world level in proportion to the added level of radiation. As the amount of genetic damage (and genetic deaths) is equal to the mutation rate,[4] these populations are continually adversely influenced by the higher mutation rate due to both external and internal radiation from fallout.

Several authors[5] have indicated that many, perhaps the majority, of chromosomes in natural populations carry lethal or detrimental factors. These are recessive in the usual sense that they have some slight detrimental effect in the heterozygotes. A very large proportion of the chromosomes of *D. willistoni*, which is the major tropical American species, carry detrimental or lethal characters. *Drosophila prosaltans*, with a much smaller population in these regions, certainly carries fewer detrimental factors. If we check the *D. hydei* tests in Figure 4 and those of the better strains of *D. novamexicana* (omitting 4 because of its serious semidominant lethal effect), it is obvious that there are comparatively few detrimental factors in the small or sparse populations. *Drosophila ananassae* carries more. To get a better idea of what the reduction below the level of the *D. hydei* or good *D. novamexi-*

cana tests implies in additional lethals, we should remember that each additional (recessive) lethal or lethal equivalent reduces the viable survivors by 25 per cent and so reduces the total egg development less and less for each additional lethal.

Another point of interest for these tests showing reduced development for 1955 by Rongelap and Bikini can be made on the following basis. Lerner[6] has presented the view held also by Dobzhansky[7] and Wallace[8] that heterozygosity increases the homeostasis and survival value, and homozygosity of detrimental factors is particularly liable to reduce survival value. Insofar as this is so, it strengthens our contention that Bikini and Rongelap in 1955 carried an excessive load of mutations because of radiation which determined their low egg-development values.

Natural populations of bisexual organisms have a reproductive pattern between the limits set by inbreeding and those by crossbreeding (random mating). If they have a satisfactory species genotype and a suitable population structure, they can occupy a favorable range like the *D. hydei* populations and some *D. novamexicana* populations (Fig. 4). With a different population structure, such as *D. willistoni*,[5] or with an increased mutation rate due to radiation, such as the Bikini and Rongelap populations and their crosses in 1955, which are comparable to the heavily and repeatedly irradiated populations studied by Wallace,[8] there is a greater attrition of the population by any inbreeding or partial dominant effects in crossbreeding.

Summary.—Genetic analyses were made of irradiated and control isolated populations of *D. ananassae* from the Pacific Proving Ground area, others of the Marshall Islands, and Ponape. These tests show that the Bikini and Rongelap populations were severely damaged genetically by the direct radiations and fallout from the thermonuclear device exploded on March 1, 1954.

The Rongerik atoll populations had recovered from fallout by August, 1955—a period roughly equivalent to 26 generations. The populations collected at Rongelap and Bikini had not recovered then, but the Rongelap population had recovered by 1956, in 40 or more generations. Because of the heavier irradiation on Bikini—including effects of the weapons tests in 1956—this population was still slightly below other Pacific Island populations even in 1957.

The general relations and comparisons between these isolated island populations and other *Drosophila* populations are discussed.

The authors gratefully wish to acknowledge the support of Mr. Ernest Wynkoop, A.E.C. resident engineer, and his staff at the Pacific Proving Ground; Mr. Maynard Neass, district administrator on Majuro; and Mr. Henry Hedges, district administrator on Ponape, as well as the logistic support of the Air Corps and Navy. We wish to express our thanks to Dr. Marvin Wasserman, Dr. Calvin Ward, Mr. Tsueng-Hsing Chang, Mr. N. B. KrishnaMurthy, and Mr. Thomas Gregg for their assistance in collecting and laboratory work.

* This work was supported by a contract with the Atomic Energy Commission [AT-(40-1)-1323] and by a grant from the Rockefeller Foundation.

[1] Wilson S. Stone, Marshall R. Wheeler, Warren P. Spencer, Florence D. Wilson, June T. Neuenschwander, Thomas G. Gregg, Robert L. Seecof, and Calvin L. Ward. *Univ. Texas Pub.*, No. 5721, p. 260, 1957.

[2] Gordon M. Dunning, *Radioactive Contamination of Certain Areas in the Pacific Ocean from Nuclear Tests* (Washington, D.C.: U. S. Atomic Energy Commission, 1957).

[3] Wilson S. Stone, Mary L. Alexander, and Frances E. Clayton, *Univ. Texas Pub.*, No. 5422, p. 272, 1954.

[4] Sewall Wright, *J. Cell. Comp. Physiol.*, **35** (Suppl. 1), 187, 1950.
[5] Theodosius Dobzhansky and Boris Spassky, *Genetics*, **39**, 472, 1954.
[6] I. Michael Lerner, *Genetic Homeostasis* (New York: John Wiley & Sons, Inc., 1954).
[7] Theodosius Dobzhansky, *Cold Spring Harbor Symposia on Quant. Biol.*, **20**, 1, 1955.
[8] Bruce Wallace, *J. Genet.*, **54**, 280, 1956.

A NEW PROOF OF THE STRONG MARKOV THEOREM OF CHUNG

BY D. G. AUSTIN*

YALE UNIVERSITY, UNIVERSITY OF MIAMI

Communicated by Einar Hille, April 22, 1958

We consider time-homogeneous Markov processes with continuous time parameter and discrete phase space. The recent important result of K. L. Chung[1] that every such process has the strong Markov property will be obtained by approximating the optional time by simple random variables (see below for a precise statement).

It should be pointed out that Chung's result includes the critical point $t=0$ in the theorem below with respect to the set where $y(0) \neq \infty$. In addition to the strong Markov theorem Chung also gives the explicit form of the conditional distribution of $y(t)$ relative to α and proves it continuous in t—a result useful in applications of the theorem (see footnote 6). I should like to thank Professor Chung and Professor Kakutani for many helpful conversations during the development of this proof.

Let $(P, \mathfrak{F}, \Omega)$ be a probability triple, where Ω is any abstract space, $\mathfrak{F}$ is a Borel field, of subsets of Ω, and P is a probability measure on $\mathfrak{F}$. We consider stochastic processes of the form $x(t, w)$, where $w \in \Omega$, t is on the non-negative real line R, and where it is always assumed that the range is contained in the space of positive integers.[2] We denote the time-homogeneous Markov processes[3] by $\mathfrak{M}$; let $\{p_{ij}(t)\}$ be a transition matrix for $x(t, w) \in \mathfrak{M}$; such a matrix will be called standard if $\lim_{t \to 0^+} p_{ij}(t) = \delta_{ij}$. When a sequence of random variables $y_n(w)$ converges in probability to $y(w)$ we write $p \lim_n y_n(w) = y(w)$.

Two conditional probabilities will be described as equal if they are equal almost everywhere $[P]$. We then state a convergence result in a form stronger than will be needed for our applications.

LEMMA. *i) Let $y(t, w)$, $y_n(t, w)$ $(n = 1, 2, \ldots)$ be stochastic processes: and suppose that $y_n(t_m, w) \to y(t_m, w)$ a.e. $[P]$ for $t_m \in R$, $m = 0, 1, 2, \ldots, k$: then if Λ is any set in $\mathfrak{F}$ and A_m $(m = 1, 2 \ldots k)$ are collections of integers, we have*

$$\lim_n P\,[\Lambda;\, y_n(t_m) \in A_m, m = 1,2, \ldots, k \,|\, y_n(t_0)] \tag{1}$$

$$= P\,[\Lambda;\, y(t_m) \in A_m,\, m = 1, 2, \ldots, k \,|\, y(t_0)].$$

ii) If the $y_n(t, w)$ are Markov processes and if for each t, $p \lim_n y_n(t, w) = y(t, w)$, then $y(t, w)$ is a Markov process.

iii) If $y_n(t, w) \in \mathfrak{M}$ *with transitions* $p_{ij}^{(n)}(t)$ *then* $y(t, w)$ *will be in* $\mathfrak{M}$ *with transitions* $p_{ij}(t)$ *and* $\lim_n p_{ij}^{(n)}(t) = p_{ij}(t)$ *for any state* i *such that* $P\ [y(\tau) = i] > 0$ *for some* $\tau > 0$.

iv) Finally, *under the conditions of the last sentence if* $p_{ij}^{(n)}(t)$ *are identical for all* n, *then* $p_{ij}^{(n)}(t)$ *will be a transition matrix for* $y(t, w)$.

Proof: We prove only (i) the remainder of the lemma will then follow readily. The proof of (*i*) will be clear if we fix an integer $\{i\}$ such that $P[y(t_0) = i] > 0$ and let $k = 1$ where A_1 is the single integer $\{j\}$ and prove

$$\lim_n P[\Lambda;\ y_n(t_1) = j]/P[y_n(t_0) = i] = P[\Lambda;\ y(t_1) = j]/P[y(t_0) = i]. \tag{2}$$

Now let ϵ be any positive number; then we may pick a number $\eta > 0$ so that for any $\Delta \in \mathfrak{F}$ with $P(\Delta) < \eta$ we have

$$P[\Lambda \cap (y(t_1) = j) \cup \Delta]/P\ [y(t_0) = i) \cap \Delta] - P\ [\Lambda \cap (y(t_1) = j) \cap \Delta]/P[(y(t_0) = i) \cup \Delta] < \epsilon. \tag{3}$$

Now by the Egorov theroem we may pick Δ and N so that $P\Delta < \eta$ and $\left|y(t_k, w) - y_n(t_k, w)\right| < {}^1/_2$ for $w \notin \Delta$, $k = 0,1$ and $n > N$. That is, for $n > N$, $w \notin \Delta$, $y_n(t_k, w) = y(t_n, w)$; hence it follows from (3) that, for $n > N$,

$$\left|P[\Lambda \cap (y(t_1) = j]/P[y(t_0) = i] - P[\Lambda \cap (y_n(t_1 = j)]/P[y_n(t_0) = i]\right| < \epsilon,$$

and (2) is proved.

We proceed to the strong Markov theorem. Let $x(t, w)$ be a time-homogeneous Markov process with standard transitions and let $\mathfrak{B}$ denote the Borel sets of R: then Chung[2] shows that $x(t, w)$ has a standard modification which is measurable $(\mathfrak{B} \times \mathfrak{F}, R \times \Omega)$ and indeed has almost all of its sample functions right lower semicontinuous in t (the standard modification may take on the value $+\infty$, but for each t this value will be assumed only in a set of probability 0). We denote by $\mathfrak{M}^*$ the class $(x(t, w); \{p_{ij}(t)\})$, where $x(t, w)$ is in $\mathfrak{M}$ and has right lower semicontinuous sample functions and where $\{p_{ij}(t)\}$ is a standard transition matrix for $x(t, w)$. If $y(t), t \in T$, $T \subset R$ is some collection of random variables, then $\mathfrak{F}(y(t);\ t \in T)$ denotes the Borel field which they generate. A random variable $\alpha(w)$ with range some subset of R will be called optional if $[w;\ \alpha(w) \leq t] \in \mathfrak{F}(x(s);\ s \leq t)$. A set Λ will be called admissible if $[\Lambda \cap (\alpha(w) \leqq t)] \in \mathfrak{F}(x(s);\ s \leqq t)$ for each t.

THEOREM. *If* $x(t, w) \in \mathfrak{M}^*$ *and* $\alpha(w)$ *is optional, then* $y(t, w) = x(\alpha(w) + t, w)$ *is a stochastically continuous process in* $\mathfrak{M}$ $(t > 0)$ *having the same transition as* $x(t, w)$ *and satisfying* $P[y(t) = \infty] = 0$, $t > 0$. *Furthermore, if* Λ *is admissible and if* $M_1 \in \mathfrak{F}(y(s):\ s < t^*)$ *and* $M_2 \in \mathfrak{F}(y(s);\ s \geqq t^*)$ *for some* t^*, *then*

$$P[\Lambda\ M_1M_2\,|\,y(t^*)] = P\ [\Lambda M_1\,|\,y(t^*)] \cdot P\ [M_2\,|\,y(t^*)]. \tag{4}$$

Proof: Let $\alpha_n(w)$ be a sequence of optional random variables having denumerable range such that $\lim \alpha_n(w) = \alpha(w)$. Then the stochastic processes $y_n(t, w) = x(\alpha_n(w) + t, w)$ converge to $y(t, w)$ in measure on $(\mathfrak{B} \times \mathfrak{F}, R \times \Omega)$; this follows from the product space version of Auerbach's theorem.

Hence, by possibly choosing a subsequence, we may suppose that $\lim_n y_n(t, w) = y(t, w)$ almost everywhere on the product space. It is clear however that $y_n(t, w)$

is in $\mathfrak{M}$ with transitions $\{p_{ij}(t)\}$ and that (4) is satisfied with $y(t, w)$ replaced by $y_n(t, w)$; to see this, one need merely note that if $\alpha_n(w) = c$ on $\Delta_c \in \mathfrak{F}(x(t); t \leq c)$ where $P(\Delta_c) > 0$, then $y(t, w) = x(t + c, w)$ is in $\mathfrak{M}$ with transitions $\{p_{ij}(t)\}$ on the probability triple $(P(\cdot \mid \Delta_c), \Delta_c\mathfrak{F}, \Delta_c)$.

We now show that $y_n(t, w)$ form a sequence of Markov processes which are equi-uniformly right continuous in probability on any finite interval $[t_1, t_2]$ where $t_1 > 0$. We pick a t^* with $0 < t^* < t_1$ such that $P[y(t^*) = \infty] = 0$ and $\lim_n y_n(t^*) = y(t^*)$ a.e. $[P]$; such a choice is possible by Fubini's theorem. Let $\epsilon > 0$ be assigned; then, using Egorov's theorem, we see that there exist positive integers M and N and a set Δ with $P(\Delta) < \epsilon$ such that for $n > N$, $w \notin \Delta$ we have $y_n(t^*) = y(t^*) < M$. Let $p_i = P[y(t^*) = i]$; then, for $n > N$ and $t > t^*$.

$$P[y_n(t) \neq y_n(t + \tau)] \leq \sum_{i=1}^{M} \sum_{j=1}^{\infty} p_i p_{ij}(t - t^*)\,[1 - p_{jj}(\tau)] + P(\Delta). \qquad (5)$$

But the elements of a regular transition matrix are continuous functions; so, in view of Dini's theorem, the double sum in (5) converges to 0 as $\tau \to 0^+$ uniformly for t in any finite interval. The uniform continuity in probability assertion is proved, and from this it follows that for each t, $y_n(t, w)$ is a Cauchy sequence in measure and the function $\tilde{y}(t, w) = p \lim_n y_n(t, w)$ is stochastically continuous, satisfying

$$P(\tilde{y}(t, w) = \infty] = 0 \text{ for each } t > 0.$$

By (*iii*) and (*iv*) of the lemma, $y(t, w)$ is in $\mathfrak{M}$ with transition matrix $\{p_{ij}(t)\}$, and by (*i*) of the lemma, (4) is satisfied with $y(t, w)$ replaced by $\tilde{y}(t, w)$. We have left only to show that $\tilde{y}(t, w)$ is a standard modification of $y(t, w)$. It follows from a theorem of Doob[4] that $y(t, w)$ has at most one finite right-hand limit point; hence if $\Omega_t = [w; \tilde{y}(t, w) \neq y(t, w)]$; then by the right lower semicontinuity of $y(t, w)$ and the stochastic continuity of $y(t, w)$, we have that $y(t, w) = \infty$ a.e. $[P]$ in Ω_t. But this implies that $P(\Omega_t) = 0$ and hence $\tilde{y}(t, w)$ is indeed a standard modification of $y(t, w)$.

The argument used here seems applicable to more general phase spaces. The arguments become more delicate however and our work in this direction is still incomplete. Professor S. Kakutani has shown us by example that the lemma is not true in its full strength even with such restrictions as continuity of the sample functions or denumerability of the phase space. Also D. Ray[5] has shown that right continuity of the sample functions is not sufficient to conclude the strong Markov property in the case where the phase space is the real line.[6]

* This work was in part supported by the U.S. Air Force under contract No. AF 49(638)-184, monitored by the Office of Scientific Research.

[1] Chung, K. L., "On a basic property of Markov chains," to appear, Annals of Math. (or see Tech. note—Statistical Research Center, Univ. of Chicago).

[2] Definitions and results not explicitly referred to here may be found in J. L. Doob, *Stochastic Processes* (New York, 1953).

[3] It should be noted that a given Markov process $x(t, w)$ may have many transitions and that the property of being time-homogeneous is not invariant under the choice of transitions. When we say that a process is time-homogeneous we mean that it has a set of stationary transitions, or more specifically that for any τ_k with $P[x(\tau_k) = i] > 0$ $(k = 1, 2)$ we have $P[x(t + \tau_1) = j \mid x(\tau_1) = i] = P[x(t + \tau_2) = j \mid x(\tau_2) = i]$ for $t > 0, j = 1, 2, \ldots$.

[4] J. L. Doob, "Topics in the Theory of Markoff Chains," *Trans. Am. Math. Soc.*, **52**, 31–64, 1942.

[5] D. Ray, "Stationary Markov Processes with Continuous Paths," *Trans. Am. Math. Soc.* ,**82**, 452–493, 1956.

[6] Added in proof. It appears that Chung's result that $y(t, w) \in \mathfrak{M}$ on $t \geqq 0$ with respect to the triple $(P(\cdot 1\Delta), \Delta F, \Delta)$ where $\Delta = \bigcup_j \Delta_j = \bigcup_j [w; x(\alpha(w), w) = j]$ will follow using our proof if one chooses the approximating $\alpha_R(w)$ more delicately. Let $R_p = [2^{-m}_{p}; m = 1, 2 \ldots]$ for $p = 1, 2 \ldots$ and let $R = \bigcup_p R_p$ be a separability set for $x(t, w)$. Since $x(t, w) \in \mathfrak{M}^*$ the set $[w; w \in \Delta_j, \alpha(w) < t] \in \mathfrak{F}_t\{x(s), s \leq t\}$. Let $\pi_p[0 = a_{1,p} < a_{2,p} < \ldots]$ be a sequence of partitions of $[0, \infty)$ with norms tending to 0 and define the optional random variables as $\alpha_p^*(w) = a_k$ on $B_k = [w; a_{k-1} < \alpha \leq a_k]$ with left inclusion at $k = 1$. Fix integers j, k and p and let $A_i = A_i(k, j) = [w; w \in \Delta_j \cap B_k, \alpha(w) < s_i, x(s_i, w) = j]$ where $s_1 < s_2 \ldots < s_q$ is an ordering of $R_p \cap (a_{k-1,p}, a_{k,p}]$. We define $\alpha_p(w) = s_l$ on $A_l - \bigcup_{l=1}^{l-1} A_1$, $l = 1, 2 \ldots q$; the r.v.'s are defined similarly for each k and j and we let $\alpha_p(w) = \alpha_p^*(w)$ elsewhere. The r.v.'s $\alpha_p(w)$ are optional and satisfy $\alpha_p(w) \downarrow \alpha(w)$ and $x(\alpha_p, w) \to x(\alpha, w)$ on Δ; hence we may let $t_1 = t^* = 0$ on the triple $(P(\cdot 1\Delta), \Delta F, \Delta)$ in the proof of our theorem and obtain the stronger result.

Finally we mention that Jushkevich has quite recently (*Russian J. of Prob.*) obtained results on the strong Markov property in a different form from that presented here. Also Chung has employed the continuity of the conditional distribution of $y(t)$ relative to α to show that $y(t)$ is separable without modification.

DYNAMIC PROGRAMMING, SUCCESSIVE APPROXIMATIONS, AND MONOTONE CONVERGENCE

By Richard Bellman

RAND CORPORATION, SANTA MONICA, CALIFORNIA

Communicated by Philip M. Morse, April 21, 1958

1. *Introduction.*—Our object in this paper is to show that a blend of dynamic programming, successive approximations, and digital computers enables us to approach various classes of variational problems formerly far beyond our reach.

To illustrate the application of these methods, we shall consider two problems. The first is that of minimizing the functional

$$J(v) = \int_0^T F(x_1, x_2, \ldots, x_N)dt + G(x_1(T), x_2(T), \ldots, x_N(T)) \tag{1.1}$$

over all forcing functions $v_i(t)$ related to the $x_i(t)$ by means of relations of the type

$$\frac{dx_i}{dt} = H_i(x_1, x_2, \ldots, x_N) + v_i(t), \qquad x_i(0) = c_i, \qquad i = 1, 2, \ldots, N, \tag{1.2}$$

and subject to the constraints of the form

$$(a)\ \int_0^T K_j(v_1, v_2, \ldots, v_N)dt \leq b_j, \qquad j = 1, 2, \ldots, L,$$

$$(b)\ p_i(t) \leq v_i(t) \leq q_i(t), \qquad 0 \leq t \leq T, i = 1, 2, \ldots, N. \tag{1.3}$$

The second problem is a generalized Hitchcock-Koopmans transportation problem. It involves the minimization of the function $C(x) = \sum g_{ij}(x_{ij})$ subject to the constraints

$$\sum_{i=1}^{N} x_{ij} = s_i, \qquad \sum_{i=1}^{M} x_{ij} = r_j, \qquad x_{ij} \geq 0. \tag{1.4}$$

As we have shown elsewhere,[1] the computational solution of questions of this type can be transformed by means of the functional equation technique of the theory of dynamic programming into problems involving the recursive determination of sequences of functions of N variables.

If $N \geq 3$, the limited memory and speed of current digital computers prevents a routine application of these techniques at the present time. Hence, if we wish to attack general problems of this nature, we must introduce more refined and subtle methods.

2. *Successive Approximation and Monotonicity.*—The starting point of our investigation is the result contained in an earlier paper,[2] where it was shown that if the equations of (1.2) are linear, and if the functions F and G are linear, then the variational problem described in the preceding section can be resolved in terms of sequences of functions of one variable, regardless of the value of N.

Furthermore, it was shown if the equations of (1.2) are linear, H is linear, but G is a non-linear function of only k of the components of $x(T)$, then a computational solution can be effected in terms of sequences of functions of k variables.

These facts lead us to contemplate the use of successive approximations; an extensive discussion will be found in Bellman.[3] Here we wish to present a simple argument which establishes the monotone character of the approximation scheme we shall discuss below. There are a number of quite interesting questions concerning convergence to the absolute minimum which we shall treat at another time.

As we have shown,[3] we can consider, by means of a suitable transformation, that F is zero. Let us consider here only the case where G is linear in the $x_i(T)$. Under these assumptions, let $v_i^{(0)}$ be a first approximation to the solution of the variational problem, and let $x_i^{(0)}$ be the state functions determined from (1.2) using these values of the $v_i^{(0)}$. To determine a second approximation, consider the problem of minimizing $G(x(T))$ over all functions satisfying the constraints of (1.3), where the x_i are related to the v_i by means of the linear equations

$$\frac{dx_i}{dt} = H_i(x_1^{(0)}, \ldots, x_N^{(0)}) + \sum_{j=1}^{N} (x_j - x_j^{(0)}) \partial H_i / \partial x_j^{(0)} + v_i(t), \qquad x_i(0) = c_i, \qquad i = 1, \ldots, N. \tag{2.1}$$

As pointed out above, this problem can be solved computationally in terms of sequences of functions of one variable. In some cases, in particular, when the constraint of (1.3a) is not present, the problem is readily solved analytically.[4, 5]

Once the new forcing functions $v_i^{(1)}$ have been obtained in this way, new state functions $x_i^{(1)}$ are determined from (1.2). Repeating this process step by step, we determine a sequence of forcing functions $\{v_i^{(k)}\}$ and a sequence of state functions $\{x_i^{(k)}\}$. Let us now show that we have monotone approximation in the sense that

$$G(x_1^{(0)}(T), \ldots, x_N^{(0)}(T)) \geq G(x_1^{(1)}(T), \ldots, x_N^{(1)}(T)) \geq \ldots \geq G(x_1^{(k)}(T), \ldots, x_N^{(k)}(T)) \geq \ldots . \tag{2.2}$$

To prove the first inequality, observe that if in (2.1), $v_i(t)$ is taken equal to

$v_i^{(0)}(t)$, we obtain a system of linear equations whose solution is clearly $x_i = x_i^{(0)}$. It follows that a set of forcing functions which minimize G subject to the linear equations of (2.1), together with the original constraints, yields a value of G which is at most $G(x_1^{(0)}(T), \ldots, x_N^{(0)}(T))$. The general result follows inductively.

This monotonicity is not surprising, since we are using the technique of approximation in policy space.[1]

3. *Successive Approximation and the Hitchcock-Koopmans Problem.*—Let us now turn to the second problem described in section 1. As a first approximation, let x_{ij} be a set of values satisfying the constraints in (1.4). To obtain a second approximation, we fix the quantities sent out from the sources $i = 3$ to $i = N$, and determine the allocations from the first two sources so as to minimize the cost of supplying the remaining demand. This problem can be resolved in terms of sequences of functions of one variable.[6]

To obtain a third approximation, we fix the allocations from the first source and the sources $i = 4$ to $i = N$, and determine the allocation from the second and third sources so as to minimize the cost of supplying the remaining demands.

Continuing in this fashion, we obtain a sequence of problems, each of whose solutions depends upon a sequence of functions of one variable. As above, it is easy to see that the sequence of costs obtained in this way is monotone decreasing. Once again, interesting questions arise concerning convergence which we do not enter into here.

Similar techniques can be applied to other classes of combinatorial problems as will be shown elsewhere.

[1] R. Bellman, *Dynamic Programming* (Princeton: Princeton University Press, 1957).

[2] R. Bellman, "Terminal Control, Time Lags, and Dynamic Programming," these PROCEEDINGS, **43**, 927–930, 1957.

[3] R. Bellman, "Some New Techniques in the Dynamic Programming Solution of Variational Problems," *Quart. Appl. Math.* (to appear).

[4] R. Bellman, I. Glicksberg, and O. Gross, "On Some Variational Problems Occurring in the Theory of Dynamic Programming," *Rend. Palermo*, Ser. II, **3**, 1–35, 1954.

[5] R. Bellman, W. H. Fleming, and D. V. Widder, "Variational Problems with Constraints," *Ann. di Mat.*, Ser. IV, **49**, 301–323, 1956.

[6] R. Bellman, "Notes on the Theory of Dynamic Programming—Transportation Models," *Management Sci.*, **4**, 191–195, 1958.

---

*ON THE THEOREM OF BERTINI FOR LOCAL DOMAINS**

BY WEI-LIANG CHOW

DEPARTMENT OF MATHEMATICS, THE JOHNS HOPKINS UNIVERSITY

Communicated by N. E. Steenrod, August 26, 1957

1. The well-known Theorem of Bertini on reducible linear systems of divisors in an algebraic variety asserts that any such system, assumed to be free from fixed components, is composite with a pencil. A somewhat special form of this theorem, which is actually a special case of it, states that if a linear system of divisors is obtained from a rational transformation of the variety onto a projective

space of dimension >1, then a generic element of the system, apart from a possible fixed component, is absolutely irreducible. In a recent discussion on local algebraic geometry, Professor S. Abhyankar raised the question whether this Theorem of Bertini, at least in the special form, also holds for local varieties, with suitable modifications. In this note we shall answer this question in the affirmative by formulating and proving such a theorem for an arbitrary complete local domain. The theorem we shall prove here can be stated as follows:

THEOREM OF BERTINI (for local domains). *Let* $(\mathfrak{R}, \mathfrak{p})$ *be a complete local domain of dimension* $t > 2$, *let* $(\mathfrak{S}, \mathfrak{q})$ *be a complete regular local domain of dimension* t *contained in* $\mathfrak{R}$ *such that* $\mathfrak{R}$ *is a finite module over* $\mathfrak{S}$ *and is separable over* $\mathfrak{S}$, *and let* x_1, x_2, x_3 *be three elements in a minimal basis for* $\mathfrak{q}$; *we set* $u(c) = (x_1 + cx_2)/x_3$, *where* c *is an element in* $\mathfrak{S}$. *Then, for sufficiently general* c, *the ideal* $\mathfrak{R}[u(c)]\mathfrak{p}$ *is prime in* $\mathfrak{R}[u(c)]$ *and the quotient ring* $\mathfrak{R}_{u(c)}$ *of* $\mathfrak{R}[u(c)]$ *with respect to* $\mathfrak{R}[u(c)]\mathfrak{p}$ *is analytically irreducible.*

We add in explanation that the expression "sufficiently general c" in the above theorem means that the $\mathfrak{q}$-residue of c is different from some one given finite set of elements in the residue field $\bar{\mathfrak{S}}$ of $\mathfrak{S}$.

The above local formulation is somewhat different in spirit from the original version of the Theorem of Bertini, in that it belongs more properly to the relative rather than to the absolute algebraic geometry. This is in a sense natural when one deals with local domains, and we have accordingly replaced the extension to algebraic closure by the extension to the completion of a local domain; furthermore, since the restriction to a local variety diminishes the degree of freedom by 1, the dimensionality condition in the local theorem has naturally to be increased by 1. Nevertheless, our proof of the local theorem will be at least formally similar to the proof given by Zariski[1] of the Theorem of Bertini in the global geometric case. Our proof is based on the following lemma, which may have some interest in itself; the discerning reader will observe that our proof of this lemma, given in the next section, is at least formally similar to the proof of Lemma 5 in the paper of Zariski quoted above, which plays a key role in his proof of the Theorem of Bertini.

LEMMA. *Let* $(\mathfrak{S}, \mathfrak{q})$ *be a complete regular local domain of dimension* $t > 2$, *and let* $x_1, \ldots, x_t$ *be the elements in a minimal basis for* $\mathfrak{q}$; *we set* $u(c) = (x_1 + cx_2)/x_3$, *where* c *is an element in* $\mathfrak{S}$, *and denote by* $\mathfrak{S}_{u(c)}$ *the quotient ring of* $\mathfrak{S}[u(c)]$ *with respect to the prime ideal* $\mathfrak{S}[u(c)]\mathfrak{q}$. *If a monic polynomial* $F(X)$ *in* $\mathfrak{S}[X]$ *(where* X *is an indeterminate) is irreducible over* $\mathfrak{S}$, *then it is also irreducible over the completion* $\mathfrak{S}_{u(c)}^*$ *of* $\mathfrak{S}_{u(c)}$ *for sufficiently general* c.

2. Before proceeding to the proof of our lemma, we shall make a few remarks on the "power-series" development of an element in the complete regular local domain $(\mathfrak{S}, \mathfrak{q})$. As before, we denote by $\bar{\mathfrak{S}}$ the residue field of $\mathfrak{S}$, and denote by Q a system of representatives of $\bar{\mathfrak{S}}$ in $\mathfrak{S}$; apart from the condition that every element in $\bar{\mathfrak{S}}$ is the $\mathfrak{q}$-residue of exactly one element in Q, the elements in Q can be chosen arbitrarily, but, for convenience, we shall assume that Q contains the elements 0 and 1. Using the properties of a regular local domain, it can easily be shown that every element in $\mathfrak{S}$ can be represented uniquely by a power series in $x_1, \ldots, x_t$ with coefficients in Q; we shall express this by setting $\mathfrak{S} = Q[[x_1, \ldots, x_t]]$. For a fixed $1 \leq j < t$, we set $v_1 = x_1/x_{j+1}, \ldots, v_j = x_j/x_{j+1}$, and denote the set $(v_1,$

$\ldots, v_j)$ by v; then $\mathfrak{S}[v]\mathfrak{q}$ is a prime ideal in $\mathfrak{S}[v]$, and the quotient ring $\mathfrak{S}_v$ of $\mathfrak{S}[v]$ with respect to $\mathfrak{S}[v]\mathfrak{q}$ is a regular local domain of dimension $t - j$, with the elements $x_{j+1}, \ldots, x_t$ as a minimal basis for its maximal prime ideal. Furthermore, the $\mathfrak{S}_v\mathfrak{q}$-residues $\bar{v}_1, \ldots, \bar{v}_j$ of $v_1, \ldots, v_j$, respectively, are independent variables over $\bar{\mathfrak{S}}$, so that the residue field of $\mathfrak{S}_v$ is a purely transcendental extension $\bar{\mathfrak{S}}(\bar{v})$ of $\bar{\mathfrak{S}}$. We denote by $Q[v]$ the set of all polynomials in v with coefficients in Q, and denote by $Q(v)$ the set of all quotients $f(v)/g(v)$ of two elements in $Q[v]$ such that the $\mathfrak{S}_v\mathfrak{q}$-residues of $f(v)$ and $g(v)$ are relatively prime (as polynomials in $\bar{v}$ with coefficients in $\bar{\mathfrak{S}}$) and that the coefficient of the highest term in $g(v)$ lexicographically (i.e., the term has the highest degree in v_1, then among these has the highest degree in v_2, etc.) is 1; then $Q(v)$ is a system of representatives in $\mathfrak{S}_v$ of its residue field $\bar{\mathfrak{S}}(\bar{v})$, and every element in the completion $\mathfrak{S}_v^*$ can be represented uniquely by a power series in $x_{j+1}, \ldots, x_t$ with coefficients in $Q(v)$, so that we can set $\mathfrak{S}_v^* = Q(v)[[x_{j+1}, \ldots, x_t]]$. Finally, given any power series in $x_{j+1}, \ldots, x_t$ with coefficients in $\mathfrak{S}[v]$, we shall define the *v-excess* (*v_i-excess, $1 \leq i < j$*) of the coefficient of any term in it to be the difference (positive or negative) between the total degree of v (the degree of v_i) in this coefficient and the degree of x_{j+1} in the term. It is easily seen that an element in $\mathfrak{S}_v^*$ is in $\mathfrak{S}$ if and only if in its power-series development the coefficient of every term is an element in $Q[v]$ of non-positive v-excess.

Proof of the Lemma: We assume that there exists an infinity of elements c in $\mathfrak{S}$, with distinct $\mathfrak{q}$-residues, such that $F(X)$ is reducible in $\mathfrak{S}_{u(c)}^*$, and we shall show that this will lead us to a contradiction. Since there is only a finite number of ways in which the polynomial $F(X)$ can split into two non-trivial monic factors, there must exist an infinite sequence of elements c_i, $i = 1, 2, \ldots$, in $\mathfrak{S}$, with distinct $\mathfrak{q}$-residues, such that we have the same factorization $F(X) = F_1(X)F_2(X)$ in each ring $\mathfrak{S}_{u(c_i)}$; we set for convenience $u_i = u(c_i)$, $i = 1, 2, \ldots$. Since the elements c_1 and c_2 have distinct $\mathfrak{p}$-residues, the elements $u_1x_3, u_2x_3, x_3, \ldots, x_t$ also form a minimal basis for $\mathfrak{p}$; in the notations introduced above, we then have the relations $\mathfrak{S}_{u_1}^* = Q(u_1)[[u_2x_3, x_3, \ldots, x_t]]$, $\mathfrak{S}_{u_2}^* = Q(u_2)[[u_1x_3, x_3, \ldots, x_t]]$, and $\mathfrak{S}_{u_1, u_2}^* = Q(u_1, u_2)[[x_3, \ldots, x_t]]$, and we observe that both $\mathfrak{S}_{u_1}^*$ and $\mathfrak{S}_{u_2}^*$ are here already canonically imbedded in $\mathfrak{S}_{u_1, u_2}^*$ as subrings. Let a be any coefficient in $F_1(X)$ or $F_2(X)$, and let $a = \sum a_{n_3 \ldots n_t} x_3^{n_3} \ldots x_t^{n_t}$ be the power-series development of a with coefficients in $Q(u_1, u_2)$. Since a is contained in $\mathfrak{S}_{u_1}^*$, it can be represented by a power series in $u_2x_3, x_3, \ldots, x_t$ with coefficients in $Q(u_1)$; it then follows from the uniqueness of the power-series representation of a that each $a_{n_3 \ldots n_t}$ is a polynomial in u_2 of degree at most n_3. Similarly, interchanging u_1 and u_2, we can show that each $a_{n_3 \ldots n_t}$ is a polynomial in u_1 of degree at most n_3. Thus we have shown that each coefficient $a_{n_3 \ldots n_t}$ is an element in $Q[u_1, u_2]$ of non-positive u_1-excess and non-positive u_2-excess. In order to prove our lemma, it is sufficient to show that the (u_1, u_2)-excess of each $a_{n_3 \ldots n_t}$ is non-positive. For, by a remark made above, the element a would then be contained in $\mathfrak{S}$, and since a is any coefficient in $F_1(X)$ or $F_2(X)$, both $F_1(X)$ and $F_2(X)$ would then be elements in $\mathfrak{S}[X]$; this would be in contradiction to our assumption that $F(X)$ is irreducible in $S[X]$.

We have the relation $u_2 = du_1 + eu_3$, where $d = (c_3 - c_2)/(c_3 - c_1)$ and $e = (c_2 - c_1)/(c_3 - c_1)$ are units in $\mathfrak{S}$; if we substitute for u_2 the expression $du_1 + eu_3$ in $a_{n_3 \ldots n_t}$, we obtain a polynomial $a_{n_3 \ldots n_t}'$ in u_1 and u_3 with coefficients in S, whose degree in u_1 is equal to the total degree of $a_{n_3 \ldots n_t}$ in u_1 and u_2. The ex-

pression $a = \sum a_{n_3} \ldots {}_{n_t}' x_3^{n_3} \ldots x_t^{n_t}$ is then a development of a as a power series in $x_3, \ldots, x_t$ with coefficients in $\mathfrak{S}[u_1, u_3]$, and if $x_3^{\mu_3} \ldots x_t^{\mu_t}$ is a term of the lowest degree such that the coefficient $a_{\mu_3} \ldots {}_{\mu_t}$ has a positive (u_1, u_2)-excess, then it is also a term of the lowest degree such that the coefficient $a_{\mu_3} \ldots {}_{\mu_t}'$ has a positive u_1-excess. We observe now that if $f(u_1, u_3)$ is any element in $\mathfrak{S}[u_1, u_3]$ and if we represent each coefficient in $f(u_1, u_3)$ by a power series in $u_1x_3, u_3x_3, x_3, \ldots, x_t$ with coefficients in Q, then we obtain a development of $f(u_1, u_3)$ as a power series in $x_3, \ldots, x_t$ with coefficients in $\mathfrak{S}[u_1, u_3]$, but the coefficient of the term of degree zero is an element in $Q[u_1, u_3]$; and it is easily seen that the u_1-excess of the coefficient of each term in this development is at most equal to the degree of $f(u_1, u_3)$ in u_1. If we now, beginning with the terms of the lowest degree, successively substitute in the power series $a = \sum a_{n_3} \ldots {}_{n_t}' x_3^{n_3} \ldots x_t^{n_t}$ the coefficients of the terms of a given degree by power-series developments of the type just described, we shall obtain a development of a as a power series $a = \sum b_{n_3} \ldots {}_{n_t} x_3^{n_3} \ldots x_t^{n_t}$ in $x_3, \ldots, x_t$ with coefficients in $Q[u_1, u_3]$, and it can be easily seen from what we have said above that the u_1-excess of $b_{\mu_3} \ldots {}_{\mu_t}$ must be positive. However, this is impossible; for the expression $a = \sum b_{n_3} \ldots {}_{n_t} x_3^{n_3} \ldots x_t^{n_t}$ is the unique development of a as a power series in $x_3, \ldots, x_t$ with coefficients in $Q[u_1, u_3]$, and by a similar argument as before with u_3 replacing u_2, we can show that the u_1-excess of each coefficient $b_{n_3} \ldots {}_{n_t}$ must be non-positive. This completes the proof of the lemma.

3. *Proof of the Theorem of Bertini:* Since $u(c)$ is the quotient of two elements in a system of parameters in $\mathfrak{R}$, it can be easily shown, by a well-known argument, that the $\mathfrak{R}[u(c)]\mathfrak{p}$-residue of $u(c)$ is a variable over $\bar{\mathfrak{R}}$, so that $\mathfrak{R}[u(c)]/\mathfrak{R}[u(c)]\mathfrak{p}$ is a polynomial ring in one variable over $\bar{\mathfrak{R}}$; this shows that $\mathfrak{R}[u(c)]\mathfrak{p}$ is a prime ideal in $\mathfrak{R}[u(c)]$ and that $\mathfrak{R}[u(c)]\mathfrak{p}$ contracts to $\mathfrak{S}[u(c)]\mathfrak{q}$ in $\mathfrak{S}[u(c)]$. In order to prove our theorem, it is sufficient to show that $\mathfrak{R}\mathfrak{S}_{u(c)}$ is an analytically irreducible local domain; for, since $\mathfrak{R}\mathfrak{S}_{u(c)}$ contains $\mathfrak{R}[u(c)]$ and is contained in $\mathfrak{R}_{u(c)}$ and since the maximal prime ideal in $\mathfrak{R}\mathfrak{S}_{u(c)}$ must then contract to $\mathfrak{R}[u(c)]\mathfrak{p}$ in $\mathfrak{R}[u(c)]$, one sees readily that $\mathfrak{R}_{u(c)} = \mathfrak{R}\mathfrak{S}_{u(c)}$. Since $\mathfrak{R}\mathfrak{S}_{u(c)}$ is a finite module over the local domain $\mathfrak{S}_{u(c)}$, it is a semilocal domain in the sense of Chevalley;[2] it is therefore sufficient to show that the completion $(\mathfrak{R}\mathfrak{S}_{u(c)})^*$ of $\mathfrak{R}\mathfrak{S}_{u(c)}$ is a local domain. Let K and L be the quotient fields of $\mathfrak{R}$ and $\mathfrak{S}$ respectively, and let L^* be the quotient field of the completion $\mathfrak{S}_{u(c)}^*$ of $\mathfrak{S}_{u(c)}$, which we recall is a regular local ring. According to a result of Chevalley,[3] $(\mathfrak{R}\mathfrak{S}_{u(c)})^*$ is a finite module over $\mathfrak{S}_{u(c)}^*$ and no non-zero element in $\mathfrak{S}_{u(c)}^*$ is a zero-divisor in $(\mathfrak{R}\mathfrak{S}_{u(c)})^*$; if we denote by $\mathfrak{M}$ the quotient ring of $(\mathfrak{R}\mathfrak{S}_{u(c)})^*$ with respect to the set of all non-zero elements in $\mathfrak{S}_{u(c)}^*$, then both K and L^* are subrings in $\mathfrak{M}$ and we have the equation $\mathfrak{M} = KL^*$ (KL^* denotes here the ring generated by K and L^*). Moreover, by the same result, every set of linearly independent elements in K over L remains such over L^*; it follows from this that $\mathfrak{M}$ is isomorphic to the tensor product $K \times L^*$ over L. Now, since K is separable over L, there exist a finite number of irreducible monic polynomials $F_i(X)$ in $\mathfrak{S}[X]$ such that any extension field L_1 of L is linearly disjoint with respect to K over L if and only if all $F_i(X)$ remain irreducible in $L_1[X]$. According to our lemma, for sufficiently general c, all $F_i(X)$ will remain irreducible in $\mathfrak{S}_{u(c)}^*[X]$; since $\mathfrak{S}_{u(c)}^*$ is integrally closed, this implies that all $F_i(X)$ will remain irreducible in $L^*[X]$. It follows that L^* is linearly disjoint with respect to K

over L, and hence the tensor product $K \times L^*$ is an integral domain; this implies of course that $(\mathfrak{R}\mathfrak{S}_{u(c)})^*$ is an integral domain. Finally, since $(\mathfrak{R}\mathfrak{S}_{u(c)})^*$ is a finite module over the complete local domain $\mathfrak{S}_{u(c)}{}^*$ and has no zero-divisors, it must be a complete local domain.[4] This completes the proof of our theorem.

We shall conclude this note by a few remarks on the hypothesis of our theorem. We observe that in our formulation of the local Theorem of Bertini, we have introduced the subring $\mathfrak{S}$ and a system of regular parameters $x_1, \ldots, x_t$ in $\mathfrak{S}$, while in the final assertion of the theorem only the three elements x_1, x_2, x_3 enter into the picture. The question naturally arises whether it is necessary at all to introduce the regular local domain $\mathfrak{S}$ or even the system of parameters $x_1, \ldots, x_t$ in $\mathfrak{R}$. As to the latter, one finds a measure of justification in the fact that, even in the equal-characteristic case where $\mathfrak{R}$ contains a coefficient field k, an arbitrary set of three analytically independent elements x_1, x_2, x_3 may generate in $\mathfrak{R}$ an ideal of rank less than 3 and hence cannot be extended into a system of parameters in $\mathfrak{R}$. This is a well-known situation where a local ring may contain as a subring a local ring of higher dimension, and one can easily obtain examples to show that the local Theorem of Bertini cannot hold for an arbitrary set of three analytically independent elements without some qualifications. As to the introduction of the regular local domain $\mathfrak{S}$, it is evidently not necessary in the equal-characteristic case, since any system of parameters $x_1, \ldots, x_t$ in $\mathfrak{R}$ can be considered as the minimal basis of the maximal prime ideal in the regular local domain $k[[x_1, \ldots, x_t]]$, where k is a coefficient field in $\mathfrak{R}$. However, in the unequal-characteristic case, we do not know whether every system of parameters in $\mathfrak{R}$ can be so imbedded in a regular local domain, and such an imbedding is essential for our present method of proof. The question therefore remains open whether the local Theorem of Bertini holds in general for any three elements in a system of parameters in $\mathfrak{R}$.

* This work was partially supported by a research grant of the National Science Foundation.

[1] O. Zariski, "Pencils on an Algebraic Variety and a New Proof of a Theorem of Bertini," *Trans. Am. Math. Soc.*, **50**, 48–70, 1941.

[2] C. Chevalley, "On the Theory of Local Rings," *Ann. Math.*, **44**, 690–708, 1943.

[3] *Ibid.*, § II, Proposition 7.

[4] *Ibid.*, § III, Proposition 8. The proof of this proposition also holds for a local ring in the sense of Krull.

THE STRUCTURE OF PRIME RINGS WITH MAXIMUM CONDITIONS

BY A. W. GOLDIE

KING'S COLLEGE, NEWCASTLE-UPON-TYNE, ENGLAND

Communicated by N. Jacobson, March 25, 1958

1. *Introduction.*—The purpose of this note is to indicate briefly the proof of the following theorem. A detailed account will appear elsewhere.

THEOREM A. *A ring R has a right and left quotient ring which is a complete matrix ring $M_n(D)$, where D is a division ring, if and only if (a) R is a prime ring; (b) R has*

maximum condition for annihilator right (and left) ideals; (c) R has maximum condition for complement right (and left) ideals.

2. *Preliminaries.*—R denotes a ring. If S is a non-empty subset of R, then set $S_r = (x \in R/Sx = 0)$. S_r is a right ideal of R, and ideals of this form are called *annihilator right ideals.* A right ideal I of R is *relatively complemented* if $I \neq 0$ and there exists a non-zero right ideal J of R with $I \cap J = 0$. The set of right ideals which contain J and have zero intersection with I has maximal elements, the *relative complements of* I. Ideals of this latter type are called *complement right ideals.* A right ideal U of R is *uniform* if $U \neq 0$ and if every pair of non-zero right ideals of R which are contained in U has non-zero intersection. An element $u \in R$ is a *right uniform element* if the principal ideal $\{u\} + uR$ is a uniform right ideal. R is a *right uniform ring* if R itself is a uniform right ideal. Corresponding definitions for left ideals are to be taken as read.

LEMMA 1. *If R has maximum condition for complement right ideals, then every non-zero right ideal I of R contains a uniform right ideal.*

Proof: If I is not uniform, then it contains non-zero right ideals I_1, J_1 with $I_1 \cap J_1 = 0$. If I_1 is not uniform, then it contains I_2, J_2 with $I_2 \cap J_2 = 0$. Proceeding in this way, one obtains two sequences of right ideals $I \supset I_1 \supset I_2 \supset \ldots$ and $J_1, J_2, \ldots$. Let I_1^c be a relative complement of I_1 such that $I_1^c \supseteq J_1$. Now $(I_2 \oplus J_2) \cap I_1^1 = 0$ implies that I_2 has a relative complement $I_2^c \supseteq (J_2 \oplus I_1^c)$ and, in particular, $I_2^c \supset I_1^c$. Proceeding thus, one obtains a strictly ascending chain of complement right ideals which terminates at I_k^c, say. Then I_k is uniform.

3. *Prime Rings.*—Throughout this section we suppose that R is a prime ring which satisfies the maximum conditions (*b*) and (*c*) of Theorem A. The following results develop the ideal structure of R.

LEMMA 2. *Let U be a uniform right ideal of R.* Then $U_l = (x \in R/x_r \cap U \neq 0)$.

COROLLARY. *For each $x \in R$ either $xU = 0$ or xU is uniform. The union of all the uniform right ideals of R is a two-sided ideal.*

THEOREM 1. *R is a right uniform ring if and only if it has no zero divisors. A right uniform ring is a left uniform ring and conversely. Such a ring has a right (also left) quotient division ring.*

If R has zero divisors, then it certainly contains a uniform right ideal U such that $U_l \neq 0$. For if a is a left zero divisor, then a_r contains a uniform right ideal. However, we can in fact prove the following result:

THEOREM 2. *Every right uniform element of R is a left uniform element of R and conversely. Every uniform right ideal has a non-zero left annihilator unless R is a right uniform ring.*

THEOREM 3. *The ring $\mathfrak{E}(U)$ of R-endomorphisms of a uniform right ideal U of R has no zero divisors, its elements other than zero are non-singular mappings, and it has a right quotient division ring.*

The set of uniform right ideals of R has maximal elements which are called *basic right ideals.* Their existence can be settled immediately by Zorn's lemma or by a longer route without transfinite methods. In fact, we have

THEOREM 4. *The basic right ideals of R coincide with the minimal annihilator right ideals of R. The right ideals u_r, where u is a (right) uniform element, coincide with the maximal annihilator right ideals.*

Direct sums of basic right ideals B_α can be formed, and condition (*c*) allows only

a finite number of terms in such a sum. The *length* of $S = B_1 \oplus \ldots \oplus B_k$ is k, and this sum has *maximum length* if the equation $S \cap I = 0$ for a right ideal I implies that $I = 0$. When this occurs, we say that S *meets all right ideals of* R.

THEOREM 5. *Direct sums of basic right ideals of R which have maximum length have the same length.*

This common maximum length is the *right dimension of* R. The left dimension of R can be defined and equals the right dimension.

Theorem 5 actually holds for any right R-module, R any ring, which satisfies a module condition corresponding to (c). It may thus be regarded as a first result in a theory of finitely generated modules.

THEOREM 6. *Every right ideal of R which is a maximal complement right ideal is also a maximal annihilator right ideal and conversely.*

Although not essential for Theorem A one can apply Theorem 6 to prove that the complement right ideals and annihilator right ideals of R coincide.

An element of R is *regular* if it is neither a left nor a right zero divisor.

THEOREM 7. *A right ideal I of R meets all right ideals of R if and only if I has a regular element.*

COROLLARY. *R has a regular element.*

These results are applied to prove that R has a right quotient ring $Q(R)$ in the sense of Jacobson.[1] The conditions on $Q(R)$ are as follows: (1) $Q(R) \supseteq R$; (2) every regular element of R has a two-sided inverse in $Q(R)$; (3) every element of $Q(R)$ has the form ac^{-1}, $a, c \in R$ and c regular. The condition given by Jacobson for the existence of $Q(R)$ can be verified in our case by means of Theorem 7, together with the fact that R has regular elements. It should be noted that R need not have an identity. Finally, one can prove that $Q(R)$ is a ring satisfying conditions (a), (b), and (c). The above theory thus applies to $Q(R)$ with the additional force that every regular element of $Q(R)$ has an inverse in $Q(R)$. This means that $Q(R)$ is a simple ring with minimum condition and hence is isomorphic to a complete matrix ring $M_n(D)$. The number n is actually the dimension of the ring R, and D is the right quotient ring of the ring $\mathfrak{E}(U)$ of R-endormorphisms of any uniform right ideal of R. The completion of the proof of Theorem A is a routine matter.

[1] N. Jacobson, *The Theory of Rings* ("Math. Surveys," Vol. II [Am. Math. Soc., 1943]).

ON THE STRUCTURE OF COMPACT RIEMANNIAN MANIFOLDS

BY W. KLINGENBERG

INSTITUTE FOR ADVANCED STUDY

Communicated by Deane Montgomery, April 4, 1958

We consider Riemannian manifolds of class C^∞.

THEOREM 1. *Let M be a compact Riemannian manifold of even dimension and everywhere positive sectional curvature K. It is well known that M then is locally irreducible, complete and either simply connected or else a 2-fold covering of M is simply connected (cf. Synge[1] and Preissmann[5]). Let L be an upper bound for K, i.e.*

$$0 < K \leq L. \tag{1}$$

If M is simply connected, the following holds:

(a) *The length of a closed geodesic is at least $2\pi/\sqrt{L}$.*

(b) *The distance between a point and its cut locus is at least $\pi/\sqrt{L}$, and hence the diameter of M is at least $\pi/\sqrt{L}$. On the other hand, if H is a positive lower bound for K, the diameter of M is at most $\pi/\sqrt{H}$.*

c) *If two points have a distance less than $\pi/\sqrt{L}$, then there exists a unique shortest geodesic segment joining them.*

If M is not simply connected, the numbers $2\pi/\sqrt{L}$, $\pi/\sqrt{L}$, $\pi/\sqrt{L}$ in (a), (b), (c) have to be replaced by half their values.

Here the cut locus $C(p)$ of a point $p \in M$ is defined as follows, cf. Whitehead.[2] On each geodesic ray g, emanating from p, one considers the set of points q' with the following property: The segment pq' of g is the unique shortest curve joining p and q'. Because M is compact, this set has an upper limit. $C(p)$ is the set of these limit points for all geodesic rays through p.

Remarks.—1. For the case dim $(M) = 2$ this theorem has been proved by Pogorelov[3] in a quite different manner.

2. The bounds given in the theorem are the best possible, as one sees from the example of the sphere with curvature L, for the simply connected case, and from the elliptic space of curvature L, for the non-simply connected case.

3. It is not known, whether the assumption dim(M) even is necessary. This assumption is used only once, and that in an application of a result of Synge, see below.

Outline of the Proof.—Obviously, it is sufficient to consider the case M simply connected. There exists a point p in M for which the distance between p and its cut locus $C(p)$ is a minimum. Assume that this minimum is $(\pi - \rho)/\sqrt{L} < \pi/\sqrt{L}$. Let q be a point on $C(p)$ which has the minimum distance from p. From (1) one sees that p and q are not conjugate, and, together with the fact that q is a point on $C(p)$ closest to p, and p is a point on $C(q)$ closest to q, one derives that there passes a closed geodesic g of length $2(\pi - \rho)/\sqrt{L}$ through p and q. Because dim(M) is even and M is simply connected, it follows from a paper of Synge,[1] that there exists a family of closed curves h, converging toward g, all of which have a length $< 2(\pi - \rho)/\sqrt{L}$. On each h we choose a point $p(h)$ such as to converge to the point p on g, if h converges toward g. The curve h does not meet the cut locus $C(p(h))$ of $p(h)$. Therefore, each point of $h - p(h)$ can be joined with $p(h)$ by a unique shortest geodesic segment. One shows that among these geodesic segments there exist one, $g(\alpha, h)$, which meets h at $p(h)$ with a given angle α, $0 < \alpha < \pi$. Furthermore, as h approaches g, the segments $g(\alpha, h)$ have as limit a geodesic segment $g(\alpha)$, which joins the point $p \in g$ with the opposite point $q \in g$ and forms with g at p the angle α. This holds for every angle α, and therefore q must be conjugate to p, which it is not. This proves part (a) of the theorem. From this, parts (b) and (c) are immediate consequences.

As an application of Theorem 1, one gets the following improved version of Rauch's sphere theorem[4] for even dimensional manifolds.

THEOREM 2. *Let M be a complete Riemannian manifold of even dimension. If the sectional curvature K of M satisfies the inequalities*

$$0 < hL < K \leq L, \tag{2}$$

where $h \sim 0.54$ *is the solution of the equation* $\sin \pi\sqrt{h} = \sqrt{h}$, *than* M *is diffeomorphic either with the sphere or with the elliptic space.*

Remark.—In Rauch's theorem the number h on the left hand of (2) is the solution of the equation $\sin \pi\sqrt{h} = \sqrt{h}/2$, i.e., $h \sim 0.74$. As one sees from the example of the complex projective space, considered as even dimensional Riemannian manifold, for $\dim(M) > 2$ the number h on the left hand of (2) cannot be smaller than 0.25.

The proof of this theorem is straightforward. It is sufficient to consider the case that M is simply connected. Fix a point p in M. Then $M - C(p)$ is an open neighborhood of p. From (2) it follows that the diameter of $C(p)$is less than $\pi/\sqrt{L}$. Therefore, if q is a point on $C(p)$, one has from theorem 1, that the open neighborhood $M - C(q)$ of q covers $C(p)$. It can be shown that, on each geodesic segment in $M - C(p)$, emanating from p, there exists exactly one point m which has the same distance, $< \pi/\sqrt{L}$, from p and q. Now one maps the north pole of the unit sphere S onto p and the geodesic segments of length $\pi/2$, starting at the north pole, onto the geodesic segments pm, starting at p. This map can be continued to a map of the southern hemisphere of S such that the south pole goes into q and the geodesic segments of length $\pi/2$, starting at the south pole, go into the segments qm, starting at q.

[1] J. L. Synge, "On the Connectivity of Spaces with Positive Curvature," *Quart. J. Math. Oxford Ser.*, **7**, 316–320, 1936.

[2] J. H. C. Whitehead, "On the Covering of a Complete Space by the Geodesics through a Point," *Ann. Math.*, **36**, 679–704, 1935.

[3] A. Pogorelov, "A Theorem Regarding Geodesics on Closed Convex Surfaces," *Mat. Sb.*, N.S., **18** (60), 181–183, 1946.

[4] H. E. Rauch, "A Contribution to Differential Geometry in the Large," *Ann. Math.*, **54**, 38–55, 1951.

[5] A. Preissmann, "Quelques propriétés globales des espaces de Riemann," *Comment. Math. Helv.*, **15**, 175–216, 1942–43.

A FORMULA FOR THE MULTIPLICITY OF A WEIGHT

By Bertram Kostant

DEPARTMENT OF MATHEMATICS, UNIVERSITY OF CALIFORNIA, BERKELEY

Communicated by Griffith Evans, April 18, 1958

1. Let $\mathfrak{g}$ be a complex semisimple Lie algebra and $\mathfrak{h}$ a Cartan subalgebra of $\mathfrak{g}$. Let π_λ be an irreducible representation of $\mathfrak{g}$, with the highest weight λ, on a finite dimensional vector space V_λ. A well-known theorem of E. Cartan asserts that the highest weight, λ, of π_λ occurs with multiplicity one. It has been a question of long standing to determine, more generally, the multiplicity of an arbitrary weight of π_λ. Weyl's formula for the character of π_λ is an expression for the function $\chi_\lambda(x) = \operatorname{tr} \exp \pi_\lambda(x)$, $x \in \mathfrak{h}$, on $\mathfrak{h}$ in terms of λ and quantities independent of the representation. In the same spirit the author has always understood the multiplicity question to mean the following: Let I be the set of all integral linear forms

on $\mathfrak{h}$. Let m_λ be the function in I which assigns to each integral linear form $\nu \in I$ the multiplicity $m_\lambda(\nu)$ of its occurrence as a weight of π_λ. Find a formula for the multiplicity function m_λ in terms of λ and quantities independent of the representation.[1]

Obviously a knowledge of the multiplicity function m_λ determines $\chi_\lambda(x)$, $x \in \mathfrak{h}$. That is

$$\chi_\lambda(x) = \sum_{\nu \in I} m_\lambda(\nu) \exp(\nu, x).$$

On the other hand, Weyl's formula asserts that

$$\chi_\lambda(x) = \frac{\sum_{\sigma \in W} sg(\sigma) \exp(\sigma(g + \lambda), x)}{\sum_{\sigma \in W} sg(\sigma) \exp(\sigma(g), x)},$$

where g is one-half the sum of the positive roots and W is the Weyl group. Finding a formula for the multiplicity function m_λ in a sense then "accomplishes" the division indicated by the formula of Weyl. We hasten to add—this does not in any way detract from Weyl's formula, since it still retains its overriding and quite remarkable feature of expressing what is, in general, a very complicated trigonometric polynomial on $\mathfrak{h}$ as a quotient of two relatively simple trigonometric polynomials.

Let $P(\mu)$, $\mu \in I$, be the integer valued function on I defined by

$P(\mu)$ = no. of ways μ may be partitioned into a sum of positive roots.

It follows from elementary considerations in representation theory that the inequality

$$m_\lambda(\lambda - \mu) \leq P(\mu) \tag{1}$$

holds for all dominant λ and all $\mu \in I$. Now one can show (and we exploit this fact) that, fixing μ, for λ sufficiently "far out" in the fundamental chamber and sufficiently far from the "walls" of the chamber, the equality sign in (1) will always hold. It seems clear, then, that a formula for m_λ must necessarily involve the function P. It is the purpose of this note to announce the

THEOREM. *The multiplicity function m_λ is given by the following formula:*

$$m_\lambda(\nu) = \sum_{\sigma \in W} sg(\sigma) P(\sigma(g + \lambda) - (g + \nu)).$$

Putting $\lambda = 0$ yields the following recursive relation for the partition function P:

COROLLARY:

$$P(\mu) = -\sum_{\substack{\sigma \in W \\ \sigma \neq e}} sg(\sigma) P(\mu - (g - \sigma g))$$

for $\mu \neq 0$, $\mu \in I$. (The recursive nature of the above formula is further clarified when it is recalled that P vanishes outside the cone generated by the positive roots and $g - \sigma g$ lies in that cone. Also $P(0) = 1$.)

[1] A device for computing the multiplicity function has been given by Freudenthal. It is based on the recursive properties of the Hauptformel; see H. Freudenthal, "Zur Berechnung der Charaktere der halbeinfachen Lieschen Gruppen. I," *Proc. Kon. Ned. Akad. Wet.*, Ser. A, **57**, pp. 369–376, 1954.

*TRANSDUCTION BY BACTERIOPHAGE P1: ABNORMAL PHAGE FUNCTION OF THE TRANSDUCING PARTICLES**

By Jane N. Adams and S. E. Luria

department of bacteriology, University of Illinois, Urbana, Illinois

Communicated by M. Delbrück, April 1, 1958

Transduction by bacteriophage, as first described in *Salmonella*,[1] is the phage-mediated transfer of any one of a number of genetic traits from one bacterial cell to another. The frequency of genetic transfer is quite low, $1:10^5$–$1:10^8$ per infecting phage particle for any one trait. Further study of this phenomenon both in *Salmonella* and in the coli-dysentery bacteria,[2, 3, 4, 5, 6] showed that closely linked genetic factors can be transduced jointly and that, although transduction can be detected only when the phage particles do not kill the host cell, lysogenization does not necessarily accompany transduction. Other pertinent facts about transduction are summarized in a recent review.[7]

In the so-called "specialized transduction" of the *Gal* factors of *E. coli* by phage λ,[8, 9] a different situation is observed. The *Gal* genetic region appears to have become intimately associated with the genetic material of the phage, which is thereby made abnormal and defective.[10, 11]

In the instances of non-specialized transduction, it has never been tested directly whether the transducing particles are normal or not—more specifically, whether they carry and transfer, in addition to the host genetic factors, a normal phage genome. The finding of non-lysogenic transduced cells provides no evidence, because most cells that give a reductive response to a temperate phage[12] segregate out phage-carrier and non-carrier cells for many generations.[13, 14]

Studying transduction by phage P1 in the coli-dysentery group, Lennox[5] found evidence of phage abnormalities in transduced bacteria: some tendency to anti-segregation, that is, negative correlation between lysogeny and transduced characters in the progeny of transduced cells, as well as the presence of apparently defective lysogens (immune, phage-non-producing cells) among cells of *Shigella dysenteriae* receiving the *Lac*$^+$ factor by transduction from *Escherichia coli*.

To investigate the P1 transduction more closely, we studied the segregation of non-phage-carrier cells in the progeny of cells of *S. dysenteriae* Sh and *E. coli* K-12 after infection with phages P1 or P1*kc*, comparing the segregation pattern in clones from unselected cells and from transduced cells. The methods used were similar to those described by Lennox.[5]

The results were immediately striking. We found that practically all unselected infected cells that do not lyse give *pure lysogenic clones*. This is true irrespective of the recipient cell, whether *Shigella* or *E. coli* K-12; of the multiplicity of infection, from 0.5 to 15; of the genetic markers in the phage, P1 or P1*kc;* and of the temperature of postinfection incubation, which affects the proportion of lytic versus lysogenic responses.[15] The clones of unselected cells were obtained either by random distribution of infected cells into tubes or by selected isolation of single cells by micromanipulation (kindly carried out for us by Dr. D. K. Fraser). The results are shown in Table 1. The only two significantly mixed clones (with comparable numbers of lysogenic and sensitive cells) were found in an experiment where a few large,

almost divided, cells were included in the series of cells selected by micromanipulation. The absence of segregation among cells infected with phage Pl was also reported by Goodgal.[16]

TABLE 1

COMPOSITIONS OF CLONES DERIVED FROM CELLS INFECTED WITH Pl OR P1*kc*

MICRODROP EXPERIMENTS

Expt. No.	Bacteria	M.o.i.	Microdrops: With 1 Cell	Microdrops: Lysed or Dead	Microdrops: Clones	Clones with Phage	Colony Counts (Range)	Plates: All Lysogenic	Plates: Mixed	Plates: All Non-lysogenic
51	*E. coli*	0.51	41	6	35	14	28–249	12	2†	0
54	*E. coli*	1.9	27	16	11	6	39–200	6	0	0
54	*Shigella*	2.1	36	13	23	21 (20 tested)	18–130	20	0	0

TUBE EXPERIMENTS (*Shigella*)

Expt. No.	M.o.i.	Tubes and Plates: Total	Tubes and Plates: With Growth	Colonies per Plate	Plates with: All Lysogenic	Plates with: Mixed	Plates with: Non-lysogenic
12	0.7	40	15	17–200	1	0	14
11	1.9	60	11	1–100	8	0	3
7	11	60	20 (15 tested)	2–100	14	1‡	0
9	15	60	9	1–115	9	0	0

* Growing bacteria were infected with phage at 37° C. for 10 minutes, kept in antiphage serum for 5 minutes, cooled to 20° C., diluted, and distributed for clone isolation either into microdrops on slides (D. K. Fraser, *Virology*, *3*, 527, 1957) or into tubes. M.o.i. = Multiplicity of infection.

When clones in microdrops were used, the droplets with a single cell were marked. The slides were held at room temperature for 2 hours, then at 37° C. for 5–6 hours. The marked droplets where cells had grown contained about 1,000 cells. Each marked droplet was transferred to a tube with 1 ml. of broth, and 0.1-ml. samples were plated for colonies. The tubes were incubated and tested for phage (= lysogeny). The plates corresponding to tubes with phage were replicated onto Pl-sensitive cells for lysogeny test. All doubtful or negative colonies in these plates were picked and retested.

When clones in tubes were used, the tubes were incubated at 20° C. for 2 hours, then at 37° C. for 3–5 hours, and samples were plated. Most or all plates with colonies were tested by replication as above.

† One plate with 69 lysogenic, 82 non-lysogenic; one with 99 lysogenic and 22 non-lysogenic. In this experiment, some of the cells in the microdrops were elongated and clearly near division. In all other experiments, such cells were excluded from the 1-cell microdrops.

‡ 78 lysogenic; 1 non-lysogenic.

TABLE 2*

TESTS FOR LYSOGENY IN *E. coli* CELLS FROM TRANSDUCED COLONIES

Expt. No.	M.o.i.	Selected Characters	Colonies Tested: Total	Colonies Tested: Lysogenic	Colonies Tested: Non-lysogenic
46	1.5	*Xyl*$^+$	5	3	2
47	0.6	*Xyl*$^+$	11	0	11
	0.006	*Xyl*$^+$	5	0	5
	Controls	*Xyl*$^+$	*41*	*0*	*41*
48	0.9	*Lac*$^+$	4	0	4
	0.006	*Lac*$^+$	4	0	4
	Controls	*Lac*$^+$	*20*	*0*	*20*
48	0.9	(*TL*)$^+$	9	1	8
	0.006	(*TL*)$^+$	2	0	2
	Controls	(*TL*)$^+$	*20*	*0*	*20*
50	2.4	*Strep*^{r}	20	13	7
	0.4	*Strep*^{r}	24	5	19
	Controls	*Strep*^{r}	*41*	*0*	*41*

* Phage grown on *E. coli* cells T^+ L^+ *Lac*$^+$ *Xyl*$^+$ *Strep*^{r} was used to transduce these markers into *E. coli* T^- L^- *Lac*$^-$ *Xyl*$^-$ *Strep*^{s}. Cells from transduced colonies were picked and tested for phage-carrier state directly and after restreaking. The *controls* for reinfection on the plates consisted of uninfected cells T^+ L^+ *Lac*$^+$ *Xyl*$^+$ *Strep*^{r} plated together with the infected ones and identified by their unselected markers.

Very different results are obtained when colonies selected for transduction are examined for lysogeny. The results of experiments on transduction by phage grown on *E. coli* K-12 No. 58-161 (Hayes No. 11: T^+L^+ *Ara*$^+$ *Az*^{r} *Lac*$^+$ *Xyl*$^+$ *Strep*^{r})

into *E. coli* K-12 W-677 (T^- $I^-Lac_1^-$ Xyl^- $Strep^s$) are shown in Table 2. Those of transduction into *S. dysenteriae* Sh are shown in Table 3. In most experiments adsorption of phage, at various multiplicities, lasted for 10 minutes; the bacteria were centrifuged and resuspended in fresh medium to remove unadsorbed phage and were plated on selective agar without added Ca^{++} and with added $M/100$ Na citrate (except for EMB agar) to reduce reinfection of bacteria by phage Pl on the plates.[17] Antiphage serum was sometimes added to the selection plates. Controls in which non-lysogenic, genetically labeled cells with the selected characters were added to the transduction plates are included in Tables 2 and 3. They showed that

TABLE 3*

TESTS FOR LYSOGENY IN CELLS OF *Shigella* SH FROM TRANSDUCED COLONIES

EXPT. NO.	M.o.i.	SELECTED CHARACTERS	COLONIES TESTED: Total	Lysogenic	Mixed	Non-lysogenic
32	3.1	Ara^+	51 (5 Az^r)	39 (3 Az^r)	12 (2 Az^r)	0
	0.13	Ara^+	37 (8 Az^r)	33 (8 Az^r)	3 (0 Az^r)	1 (0 Az^r)
	0.01	Ara^+	75 (5 Az^r)	5 (1 Az^r)	0	10 (4 Az^r)
33	3.0	Ara^+	30 (2 Az^r)	19 (2 Az^r)	11 (0 Az^r)	0
	0.04	Ara^+	29 (6 Az^r)	6 (0 Az^r)	9 (0 Az^r)	14 (6 Az^r)
	Controls	Ara^+ Az^r	*51 (51 Az^r)*	*5 (5 Az^r)*	*26 (26 Az^r)*	*20 (20 Az^r)*
32†	3.1	Lac^+	25	2	..	23
	0.13	Lac^+	22	5	..	17
	0.01	Lac^+	16	1	..	15
33†	3.0	Lac^+	22	5	..	17
	0.04	Lac^+	20	5	..	15
41†	0.05	Lac^+	11	1	..	10
7‡	16	$Strep^r$	123	123	0	0
8‡	8	$Strep^r$	146	146	0	0
11‡	8	$Strep^r$	139	139	0	0
14‡	2	$Strep^r$	64	64	0	0
11, 14‡	*Controls*	$Strep^r$	*51*	*1*	*0*	*50*
56§	0.3	$Strep^r$	95	77	..	18
	Spontaneous mutants		(11)	(6.5)	..	(4.5)
	Controls	$Strep^r$	*24*	*0*	..	*24*

* Methods as in Table 2. Note the extensive reinfection of Ara^+ cells, which probably accounts for all mixed lysogenic clones.

† Most of the Lac^+ are unstable and segregate Lac^- cells. Most of the Lac^+ non-lysogenic are Pl-immune, but segregate Pl-sensitive, Lac^- progeny.

‡ The results of these experiments include tests on colonies from platings on streptomycin agar at various times. After infection, the phage was removed by washing, and the cells were diluted and incubated in fresh broth at 37° C. Platings were made on streptomycin agar between 2 and 8 hours after infection. The *controls* were uninfected $Strep^r$ Lac^+ cells mixed with the infected cells after washing.

§ In this experiment infected cells were spread on agar without streptomycin, overlayed with a thin agar layer, and incubated for 2 hours at room temperature and 2 hours at 37° C.; then a third agar layer was added containing streptomycin. The spontaneous mutants (figures in parentheses) were obtained from parallel plates using bacteria infected with non-transducing phage. The controls were uninfected $Strep^r$ Lac^+ cells plated together with the infected cells.

reinfection was effectively prevented when the recipient strain was *E. coli*, less so with strain Sh on EMB sugar agar (see also Lennox[5]).

Transduction for streptomycin resistance, where the selection must be applied after the new character has expressed itself, was done either by plating the infected cells after 2–11 generations of growth in broth with citrate or by plating on citrated agar and overlaying with streptomycin agar 2–4 hours later.

With most selected factors, the colonies showing the transduced characters on the selective medium were picked into citrated broth without added Ca^{++}, immediately restreaked onto the same selective medium, then grown in broth to visible turbidity. Tests for lysogeny and for immunity to phage Pl *vir* were made both on the broth culture and on 2–10 colonies from the streak. Further streaks and tests were made when required.

The frequencies of transduction (transduced cells/adsorbed phage) ranged from 1×10^{-8} to 1×10^{-6} for different characters, in agreement with previous reports.[5]

Concerning lysogeny, the main results can be summarized as follows: For most characters, whenever the transducing phage is used to infect bacteria at multiplicities of infection $\ll 1$, the majority and often the totality of the transduced clones consist only of Pl-sensitive, non-phage-carrier cells. This is true for recipient cells both of *E. coli* K-12 and of *Shigella* Sh. In cells where the Az^r character was transduced jointly with Ara^+, similar relations with lysogeny were obtained as when Ara^+ was transduced alone. At higher multiplicities of infection, a higher proportion of the transduced cells are lysogenic. Although the samples of colonies tested for each character are small, the over-all pattern shown in Tables 2 and 3 is uniform enough and different enough from that shown in Table 1 to make the conclusion quite unequivocal that transduction by single Pl particles is generally not accompanied by lysogenization.

The two exceptions are: transduction of $Strep^r$ and of Lac^+ into Sh. Most, and possibly all, of the Sh cells that have received the character $Strep^r$ are Pl-lysogenic. This may not be very significant, however, because transduction of $Strep^r$ could be studied only at relatively high multiplicities of infection.

The Sh cells receiving the Lac^+ character are either Pl-lysogenic, or, more frequently, carriers of an apparently defective Pl, conferring immunity but not lysogeny. Both the immune state and the Lac^+ character are often unstable in the transduced cells. A more complete analysis of the Lac^+ transduction is in progress and will be published later.

The present data permit us to conclude that the introduction of transduced factors by a single Pl particle into a recipient cell is generally not accompanied by the formation of phage-carrier cells. Three explanations may be proposed: either most transducing particles do not carry a phage genome; or they carry an abnormal phage genome incapable of establishing lysogeny; or there is an exclusion between establishment of transduced factors and of phage genome in the same cell, for example, a selective establishment of transduced factors in occasional, rare non-lysogenic segregants. The fact that multiple infection leads to frequent lysogenization of the transduced cells speaks against the last explanation. Also, the finding of unstable Lac^+, defective-Pl carriers in Sh suggests that the phage genome in Lac^+ transducing Pl particles may be abnormal. Attempts to detect Pl markers by superinfecting the transduced, Pl-sensitive cells with Pl mutants are in progress. The situation encountered in transduction of the $Strep^r$ factor, if confirmed by experiments with low multiplicity of infection, might bespeak a peculiar relation between this factor and the phage genome.

Our results extend the finding of joint transduction of linked characters to infection with very low multiplicities of transducing phage, providing further proof of the reality of joint transfer by the same transducing particle.

* Aided by grants from the American Cancer Society, Inc., and from the National Institute of Allergy and Infectious Diseases, U.S. Public Health Service (Grant E-1807).

[1] N. D. Zinder and J. Lederberg, *J. Bacteriol.*, **64**, 679, 1952.

[2] N. D. Zinder, *J. Cell. Comp. Physiol.*, **45** (Suppl. 2), 23, 1955.

[3] B. A. D. Stocker, N. D. Zinder, and J. Lederberg, *J. Gen. Microbiol.*, **9**, 410, 1953.

[4] M. Demerec, *Cold Spring Harbor Symposia Quant. Biol.*, **21**, 113, 1956.

[5] E. S. Lennox, *Virology*, **1**, 190, 1955.

[6] F. Jacob, *Virology*, **1**, 207, 1955.

[7] P. E. Hartman, in *Symposium on the Chemical Basis of Heredity*, ed. W. D. McElroy and B. Glass (Baltimore: Johns Hopkins Press, 1957), p. 408.

[8] M. L. Morse, E. M. Lederberg, and J. Lederberg, *Genetics*, **41**, 142, 1956.

[9] M. L. Morse, E. M. Lederberg, and J. Lederberg, *ibid.*, **41**, 758, 1956.

[10] A. Campbell, *Virology*, **4**, 366, 1957.

[11] W. Arber, G. Kellenberger, and J. Weigle, *Schweiz. Z. allgem. Pathol. u. Bakteriol.*, **20**, 659, 1957.

[12] A. Lwoff, *Bacteriol. Revs.*, **17**, 269, 1953.

[13] M. Lieb, *J. Bacteriol.*, **65**, 642, 1953.

[14] M. Levine, *Virology*, **3**, 22, 1957.

[15] G. Bertani and S. J. Nice, *J. Bacteriol.*, **67**, 202, 1954.

[16] S. H. Goodgal, *Biochim. et Biophys. Acta*, **19**, 333, 1956.

[17] G. Bertani, *J. Bacteriol.*, **62**, 293, 1951.

[18] D. K. Fraser, *Virology*, **3**, 527, 1957.

THE FUNCTION OF EXTRACELLULAR ENZYMES OF DUTCH ELM DISEASE PATHOGEN

BY AKHTAR HUSAIN AND A. E. DIMOND

Department of Plant Pathology and Botany, Connecticut Agricultural Experiment Station, New Haven

Communicated by James G. Horsfall, and read before the Academy, April 28, 1958

INTRODUCTION

The wilts comprise a great group of diseases of plants. They attack such plants as asters, bananas, cotton, elm, maple, oak, tomatoes, and zinnias. It is common knowledge that a wilted plant suffers from dehydration. Does the plant wilt in these diseases because its root cannot absorb enough water, because the stem cannot transport the water fast enough to leaves, or because the leaves cannot retain their share of the water?

In our laboratory we have investigated various mechanisms that could account for the symptoms of the wilt diseases[1] and have explored the possibility of therapeutants to eliminate or ameliorate the symptoms.[2, 3] Our data suggest that plants suffering from wilt diseases absorb adequate amounts of water from the soil but that the conduction of water through stem and petioles is reduced markedly.[4] The water-conducting tubes are usually discolored in wilt diseases, and obstructions to water flow can be seen in the form of gummy occlusons or of tyloses in some hosts. The amount of fungus that grows in these vascular tubes is usually insufficient to account for the reduced water flow.[5] Obstruction of the water-conducting vessels has been ascribed to partially hydrolyzed polysaccharides of the host cell walls as a result of the action of extracellular enzymes produced by the pathogen. For *Fusarium* wilt of tomato, pectic enzymes have been assigned an important role in this process.[6, 7, 8] In the case of Dutch elm disease, Beckman has extended these ideas to include cellulase as well.[9] In this paper we shall explore the nature of some extracellular, hydrolytic enzymes of the Dutch elm disease pathogen, *Ceratocystis ulmi* (Buisman) Moreau, examine the role they plant in the metabolism of

the fungus and in the pathogenesis of the elm tree, and explore inhibitors of these enzymes as chemotherapeutants.

METHODS

Ceratocystis ulmi (Buisman) Moreau, the fungus causing Dutch elm disease, was grown on a liquid medium containing 1.5 gm. KH_2PO_4, 0.01 gm. $FeCl_3$, 1 gm. yeast extract, 2 gm. asparagine, and 15 gm. glucose per liter of distilled water. Other carbon sources were substituted singly in the medium for glucose in experiments described below. These were citrus pectin,[10] carboxy methyl cellulose,[11] cotton cellulose,[12] and wood cellulose.[13] The fungus was also grown on a medium consisting of 250 gm. of chopped elm twigs in 1000 ml. of distilled water.

Flasks containing sterilized nutrient medium were inoculated with a highly pathogenic black strain of *C. ulmi* and were incubated on a shaker at 25° C. At the end of the growth period, cultures were filtered to remove cells, the filtrate was centrifuged, and the supernatant was retained. This is called the "culture filtrate."

The polygalacturonase activity of the culture filtrate was determined by the loss of viscosity of pectin substrates and by the appearance of reducing groups as the substrate was hydrolyzed. The substrate consisted of a 1.2 per cent solution of citris pectin or of sodium polypectate, buffered to pH 5.5 with citrate. The viscosity was measured in a size 300 Ostwald-Fenske viscometer at 32° C.

Reducing groups were analyzed by the Willstator-Schudel hypoiodite method as modified by Jansen and MacDonnell.[14] One volume of culture filtrate was incubated with 9 volumes of substrate, and aliquots were withdrawn for analysis from time to time. As controls, a boiled sample containing PG, Pectinol 100D,[15] were incubated with substrate and analyzed for reducing groups.

Cellulase activity was detected viscosimetrically, using a substrate consisting of 0.5 per cent CMC-70[11] buffered to pH 5.5 with citrate. The increase in reducing sugars from the action of cellulase was determined by Nelson's modification[16] of the micromethod of Somogyi. One milliliter of culture filtrate was added to 9 ml. of CMC-30[11] in 0.02 *M* acetate buffer, so that in the reaction mixture the pH was 5.5 and the concentration of substrate was 0.5 per cent. The reaction mixture was incubated at 35° C. One-milliliter aliquots were removed at the beginning and end of 2 hours, and enzyme activity was measured in terms of the increase in reducing sugar in this period. Each test was run in triplicate, and a boiled sample of culture filtrate and a 0.25 per cent solution of a commercial cellulase, 19 AP[15], were used with substrate as controls.

In determining the properties of the polygalacturonase of *C. ulmi*, a sample of partially purified enzyme was prepared from the culture filtrate. Five liters of culture filtrate from a week-old culture on pectin were concentrated to 500 ml. *in vacuo* at 30° C. Three volumes of cold acetone were added, and the precipitate, after settling overnight at 5° C., was collected by centrifuging and was dried under vacuum. A 1 per cent solution of the dried precipitate was dialyzed against distilled water for 12 hours, and the dialyzed solution was used as the enzyme sample.

Cellulase was partially purified, using a week-old culture of the fungus on elm twigs and yeast extract. The culture filtrate was concentrated tenfold *in vacuo* at 30° C. After centrifuging to remove cells and debris, the supernatant was mixed

with four volumes of saturated ammonium sulfate solution. The precipitate, after standing for 12 hours at 20° C., was collected by centrifugation. The precipitate was then shaken with 200 ml. of distilled water for 48 hours and contained 75 per cent of the initial cellulase activity. This was the cellulase enzyme sample.

RESULTS AND DISCUSSION

Polygalacturonase.—Polygalacturonase production was related to the carbon source on which the fungus grew. Activity was at a maximum in 4–7-day culture filtrates when the fungus grew on autoclaved elm twigs, a medium containing native and not derived pectin. Activity was moderate when the fungus grew in a nutrient containing 1 per cent pectin and was very week when the medium contained glucose alone. In all cases the polygalacturonase activity was considerably less than is shown by other pectolytic fungi.[7] The enzyme of *C. ulmi* caused a slow loss of vis-

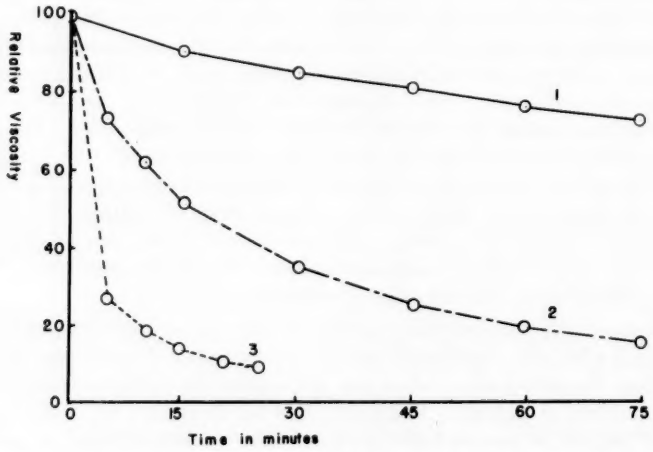


FIG. 1.—Loss of viscosity of solutions of pectin (curve *1*), sodium pectate (curve *2*), or of carboxy methyl cellulose (curve *3*) produced by culture filtrates of *C. ulmi*.

cosity of sodium polypectate but was less effective on citrus pectin (Fig. 1). When 10 ml. of active filtrate were added to 90 ml. of 0.5 per cent solution of sodium polypectate, only a small amount of reducing groups was formed, even after 12 hours of incubation (Fig. 2).

Characteristics of the polygalacturonase of *C. ulmi* include the pH at which activity is at a maximum, a property used in subsequent experiments with this enzyme. The activity of purified polygalacturonase was determined by measuring the rate of loss of viscosity of sodium polypecturate, buffered with citrate from pH 4.0 to pH 7.0 or with borate at pH 8.0. Enzyme and substrate were mixed, and the viscosity measured at stated intervals of time. Enzyme activity was expressed as the reciprocal of the time required for a 50 per cent loss in viscosity of a 1.2 per cent sodium polypectate solution. The enzyme was active from pH 4.0 to pH 8.0, with an optimum around pH 6.0 (Fig. 3).

Pectic enzymes differ in the extent to which they hydrolyze pectin. Polygalacturonase characteristically hydrolyzes pectins to galacturonic acid. Pectin depolymerase, on the other hand, hydrolyzes pectin less completely, and the products are of moderately large molecular weight. Galacturonic acid is not formed in this

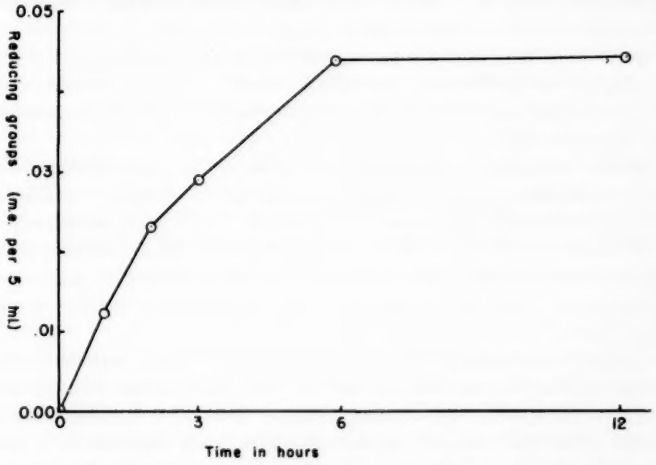


FIG. 2.—The appearance of reducing groups from sodium polypectate produced by culture filtrates of *C. ulmi*.

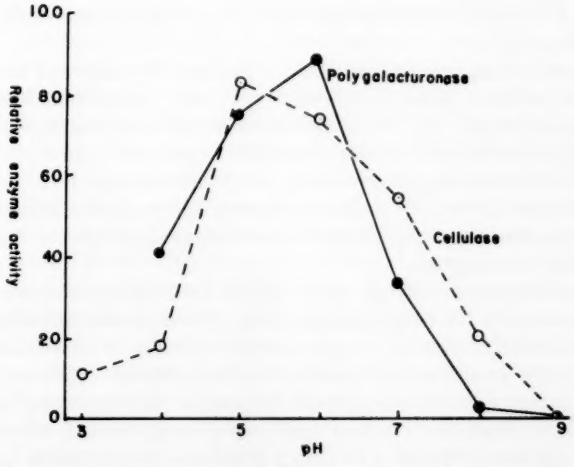


FIG. 3.—Influence of pH on the activity of polygalacturonase and cellulase of *C. ulmi*.

case. Inasmuch as pectin depolymerase has been found in culture filtrates of wilt-inducing fungi,[6, 8, 9] the hydrolytic products of the action of the present pectic enzyme were of interest. For this reason the concentrated pectic enzyme was mixed at optimum pH with sodium polypectate and incubated aseptically. Aliquots of

the reaction mixture were removed after 12, 24, and 48 hours. The products of hydrolysis were determined chromatographically, using D-galacturonic acid and the hydrolyzate from the action of 1 per cent Pectinol 100D on sodium polypectate as standards. Chromatograms were run for 20–24 hours in a solvent of butanol–acetic acid–water (4:1:5). Dried chromatograms were sprayed with aniline hydrogen phthalate[17] and heated at 105° C. for 5–10 minutes. Galacturonic acid was detected after 12 hours of incubation. However, the principal product of hydrolysis, having a slightly lower *Rf*-value, is probably the di- or trimer of galacturonic acid. Evidently the present enzyme has the characteristics of polygalacturonase and not of pectin depolymerase.

These results do not agree with those of Beckman,[9] who concluded that the fungus secretes pectin depolymerase but no polygalacturonase. Possibly he failed to detect galacturonic acid on chromatograms because the concentration of enzyme in his culture filtrates was very low. Thus even Pectinol 100D, an enzyme rich in polygalacturonase activity, does not produce detectable amounts of galacturonic acid when diluted to an activity comparable with that found in culture filtrates of *C. ulmi*.

If the polygalacturonase is active in pathogenesis, a part, perhaps a major part, of the water shortage in the diseased elm tree can be overcome through using polygalacturonase inhibitors as chemotherapeutants. Inhibitors were sought among 137 compounds. The chemical and enzyme were incubated together for 1 hour before they were added to the substrate. The concentration of the inhibitor with the enzyme was 0.1 per cent, and after mixture with the substrate the inhibitor concentration was 0.016 per cent. The index of activity of the reaction mixture was the reciprocal of the time for 50 per cent loss in viscosity when sodium polypectate was the substrate.

Inhibitors of polygalacturanase from *C. ulmi* and the degree of inhibition they caused were *t*-tridecylazomethine,[15] 49.5 per cent; potassium-2-benzothiazolyl thioglycolate,[15] 41.0 per cent; sodium-2-thiazolyl dithiocarbamate,[15] 36.2 per cent; bis dodecenyl dimethyl ammonium chloride,[15] 23 per cent. It is interesting that potassium-2-benzothiazolyl thioglycolate, which is a moderately successful chemotherapeutant experimentally,[3] is also moderately active against polygalacturonase, whereas 8-quinolinol benzoate, another chemotherapeutant for Dutch elm disease,[2] does not inhibit this enzyme.

Cellulase.—Cellulase production by *C. ulmi* was related to the source of carbon in the nutrient on which the fungus was growing. Maximal amounts of cellulase were produced on media containing carboxy methyl cellulose or autoclaved elm twigs. Cellulase was also produced on nutrient containing glucose. Culture filtrates from the former nutrients caused rapid loss in viscosity of carboxymethyl cellulose solution (Fig. 1). Culture filtrates produced only a small amount of reducing sugar when incubated with CMC-30. Thus in 2 hours the culture filtrate produced only 0.07 mg glucose ml, whereas 0.25 per cent of a commercial cellulase (19AP) produced 0.56 mg glucose/ml.

To detect the ability of the fungus to degrade native cellulose, it was grown on filter-paper strips and suspended in nutrient from which glucose was omitted. Growth of the fungus was slight, and the filter paper did not disintegrate after 1

month. Under similar conditions, *Fusarium oxysporum* f. *lycopersici*, the fungus causing tomato wilt, disintegrated paper strips within 1 week. In the case of *C. ulmi*, only weak cellulase activity was detected in the filtrate when the fungus had grown for 2 weeks on a medium containing filter-paper cellulose. Little or no growth occurred when the fungus was offered cotton or wood cellulose as a carbon source.

Our data confirm Beckman's conclusion that *C. ulmi* produces a cellulase in small amounts.[9] The breakdown of native cellulose occurs in at least two stages. The cellulose molecule has cross-linkages between polyanhydroglucose chains. Breaking of these linkages to linear chains involves C_1 activity, whereas the further hydrolysis to small molecules involves C_x activity.[18] Thus organisms that utilize carboxy methyl cellulose but not native cellulose produce the C_x enzyme. On the basis of swelling of cotton fibers, Beckman concluded that *C. ulmi* produces the C_1 enzyme. However, according to Reese and Gilligan,[19] the swelling factor is not a part of C_1 but rather of C_x. The cellulase of *C. ulmi*, therefore, appears to have C_x activity only.

To determine the pH characteristics of the concentrated cellulase from *C. ulmi*, the enzyme was incubated with CMC-70 in buffers at differing pH values in a manner comparable to that described for polygalacturonase. The enzyme was active from pH 2.0 to pH 8.0, with an optimum around pH 5.0 (Fig. 3).

Are the cleavage products of enzymatic hydrolysis of cellulose large enough to cause blockage of water-conducting vessels in the elm tree? To determine this, the cellulase of *C. ulmi* was incubated with carboxy methyl cellulose (CMC-30) at 35° C., and the hydrolytic products were determined chromatographically. One volume of concentrated enzyme was added to 9 volumes of 0.5 per cent CMC-30, and 10-ml. aliquots were removed from time to time. Samples were spotted on filter paper together with standards consisting of cellobiose and glucose. Chromatograms were produced by the methods already described for polygalacturonase. The principal product of hydrolysis was glucose, even after 6 hours of hydrolysis. Small amounts of cellobiose and other reducing substances of higher molecular weight were also detected. Levinson, Mandels, and Reese[20] reported the same result, using the cellulases of *Myrothecium verrucaria* and some other cellulolytic fungi.

Evidently, the cellulase of *C. ulmi* does not convert cellulose into products likely to clog the xylem vessels of the diseased elm tree. A number of compounds are known to inhibit the cellulases of other fungi. The activity of some of these compounds as inhibitors was measured, using the cellulase of *C. ulmi*, to determine the similarity and difference between this and other cellulases. Methods of measuring inhibition were similar to those used with polygalacturonase. Silver and mercuric ions in 10^{-3} *M* concentration almost completely inhibited the enzyme, but ions of Mn, Cu, Zn, and Fe did not. The cellulase of *M. verrucaria*[21] and those of three saprophytic fungi[22] behave similarly. Although sodium ethylene bisdithiocarbamate inhibited the cellulase of *M. verrucaria*,[21] it was ineffective against the cellulase of *C. ulmi*. 8-Hydroxyquinoline sulfate, which inhibits the cellulase of *Aspergillus oryzae*,[23] is ineffective, even at high concentrations, against the cellulase used in these studies. Sodium pentachlorophenate and 4-chloro-2-phenyl phenol in 0.1 per cent concentration were moderately active as inhibitors. Potassium-2-

benzothiazolyl thioglycolate and 8-hydroxyquinoline benzoate, both of which have been used as chemotherapeutants for Dutch elm disease,[2, 3] did not inhibit this enzyme.

Role of Enzymes in Pathogenesis.—To determine the role of polygalacturonase and cellulase in pathogenesis of *C. ulmi*, solutions of cellulase and polygalacturonase were introduced into elm cuttings.

Since *C. ulmi* produced only small amounts of polygalacturonase, a highly active purified polygalacturonase from another source[24] was used on cuttings. Fifteen milliliters of 0.05 per cent solution of polygalacturonase, containing 100 times more enzyme activity than the culture filtrate, were placed in these tubes in a constant-temperature room at 25° C. at approximately 50 per cent relative humidity. A sample of heat-inactivated enzyme was used as a control. No wilting or any external injury was observed, even after 48 hours, in any of the cuttings, although the experiment was replicated five times. Transverse sections from cuttings revealed occasional red-stained plugs whether cuttings were treated with active polygalacturonase or with heat-inactivated enzyme. Thus the deposits do not arise from enzymatic activity. In cuttings treated with active polygalacturonase, slight disorganization of vascular elements was evident. Some browning of xylem vessels and adjacent parenchyma also occurred in the areas where cells were separated by enzyme action.

A partially purified cellulase from *C. ulmi*, containing twice as much activity as the culture filtrate, was tested on cuttings in the manner described above. No external injury occurred on cuttings from the cellulase, and freehand transverse sections of the cuttings revealed no injury at the end of 48 hours of treatment.

These experiments with elm cutting suggest that pectic enzymes of *C. ulmi* play no direct role in wilting of trees or in plugging xylem vessels, in the manner postulated for *Fusarium* wilt of tomato by Gothoskar *et al.*[6] and Pierson *et al.*[25] However, the production of pectic and cellulolytic enzymes may be important in providing food for the fungus in the host. It seems logical to assume that extracellular enzymes act on the pectic and cellulosic constituents of the cell walls of the host and that the breakdown products of these large molecules are made available for the nutrition of the pathogen. Possibly the cellulase of the fungus cannot hydrolyze the crystalline part of the cellulose but acts on other noncellulosic polysaccharides having 1–4β linkage, such as xylan. Thus the cellulase from *M. verrucaria* causes hydrolysis of the xylan of wheat straw with its β1–4 xylopyranoside units.[26] The stimulation of enzyme secretion in the presence of elm wood suggests that these enzymes act upon constituents of elm wood in the diseased tree.

The search of inhibitors of polygalacturonase and of cellulase may be helpful in this connection. Inhibition of these enzymes could lead to an understanding of the role that the enzymes play in pathogenesis and to alleviation of disease severity.

SUMMARY

Polygalacturonase and cellulase are produced by the fungus causing Dutch elm disease, *C. ulmi* (Buisman) Moreau. The enzymes are extracellular, and their possible role in pathogenesis in the elm tree has been examined. The pectic enzyme is polygalacturonase and not pectin depolymerase, as shown by the appearance of reducing groups when pectins are incubated with the enzyme and by the appearance

of galacturonic acid among the hydrolytic products. A cellulase is produced that is unable to attack native cellulose, but it hydrolyzes carboxy methyl cellulose to glucose. Both enzymes are most abundantly produced by the fungus when it grows on chopped elm twigs, and both show maximum activity in the range of pH about 5.5. Apparently neither enzyme is involved in pathogenesis in the elm tree because neither produces wilting of leaves of elm cuttings subjected to the enzymes, nor are the water-transporting cells of such cuttings plugged with hydrolytic products of enzyme action upon host cell walls. At most, the enzymes enable the fungus to obtain food from host cell walls. Mercuric ion inhibits the cellulase and N-*t*-tridecylazomethine inhibits the polygalacturonase. Neither enzyme is affected by 8-quinolinol benzoate, a chemotherapeutant for Dutch elm disease. Interestingly enough, another chemotherapeutant, potassium-2-benzothiazolyl thioglycolate, inhibits the polygalacturonase but not the cellulase of *C. ulmi*.

[1] A. E. Dimond, *Ann. Rev. Plant Physiol.*, **6**, 328, 1955.

[2] A. E. Dimond, G. H. Plumb, E. M. Stoddard, and J. G. Horsfall, *Conn. Agr. Exptl. Station Bull.*, **531**, 1949.

[3] A. E. Dimond, David Davis, R. A. Chapman, and E. M. Stoddard, *Conn. Agr. Exptl. Station Bull.*, **557**, 1952.

[4] A. E. Dimond and P. E. Waggoner, *Phytopathology*, **43**, 619, 1953.

[5] P. E. Waggoner and A. E. Dimond, *Am. J. Botany*, **41**, 637, 1954.

[6] S. S. Gothoskar, R. P. Scheffer, J. C. Walker, and M. A. Stahmann, *Phytopathology*, **45**, 381, 1955.

[7] P. E. Waggoner and A. E. Dimond, *Phytopathology*, **45**, 79, 1955.

[8] R. P. Scheffer, S. S. Gothoskar, C. F. Pierson, and R. P. Collins, *Phytopathology*, **46**, 83, 1956.

[9] C. H. Beckman, *Phytopathology*, **46**, 605, 1956.

[10] Supplied by Sunkist Growers, Inc.

[11] Two types of carboxy methyl cellulose were used in this study. CMC-30 is low in substitution and low in viscosity relative to CMC-70. Supplied by Hercules Powder Co.

[12] Supplied by Hercules Powder Co.

[13] Supplied as Solka Floc by Brown Paper Co.

[14] E. F. Jansen and L. R. MacDonnell, *Arch. Biochem. Biophys.*, **8**, 97, 1945.

[15] Supplied by Rohm and Haas Co.

[16] N. Nelson, *J. Biol. Chem.*, **153**, 375, 1944.

[17] S. M. Partridge, *Nature*, **164**, 443, 1949.

[18] E. T. Reese, R. G. H. Siu, and H. S. Levinson, *J. Bact.*, **59**, 485, 1950.

[19] E. T. Reese and W. Gilligan, *Textile Research J.*, **24**, 663, 1954.

[20] H. S. Levinson, G. R. Mandels, and E. T. Reese, *Arch. Biochem. Biophys.*, **31**, 351, 1951.

[21] S. N. Basu and D. R. Whitaker, *Arch. Biochem. Biophys.*, **42**, 12, 1953.

[22] E. T. Reese and M. Mandels, *Research Rept., Pioneer Research Div., Quartermaster Research Dev. Center, Microbiol. Ser.*, Vol. **17**, 1957.

[23] M. A. Jermyn, *Australian J. Sci. Research*, **B5**, 409, 1952.

[24] Supplied through the courtesy of Dr. E. F. Jansen, Western Utilization Research Branch, Albany, California.

[25] C. F. Pierson, S. S. Gothoskar, J. C. Walker, and M. A. Stahmann, *Phytopathology*, **45**, 524. 1955.

[26] C. T. Bishop and D. R. Whitaker, *Chemistry & Industry*, **1955**, 119, 1955.

*NOMENCLATURE OF THE ACTINOMYCINS**

SELMAN A. WAKSMAN, EDWARD KATZ, AND LEO C. VINING†

INSTITUTE OF MICROBIOLOGY, RUTGERS, THE STATE UNIVERSITY, NEW BRUNSWICK, NEW JERSEY

Communicated April 18, 1958

Actinomycin, discovered by Waksman and Woodruff in 1940,[1] was the first antibiotic to be isolated in crystalline form from a culture of an actinomycete (*Streptomyces antibioticus*). Waksman *et al.*[2] found that it was a red-pigmented substance, highly active against Gram-positive bacteria and, to a lesser degree, Gram-negative organisms; it proved to be extremely toxic to experimental animals. Waksman and Tishler[3] reported that actinomycin was optically active ($[\alpha]_D^{25} = -320° \pm 5°$), possessing a molecular weight of about 768–1,000, and gave a melting point of 250° C. It exhibited characteristic absorption in the visible (450 mμ) and ultraviolet (between 230 and 250 mμ) regions. On the basis of the analytical data, an approximate empirical formula, $C_{41}H_{56}O_{11}N_9$, was suggested. Actinomycin was thought to be a polycyclic nitrogen compound, possessing a quinonoid system. Initially, it was believed that actinomycin represented two active substances, which were designated A and B. Subsequently it was discovered that the B fraction consisted of lipoidal material contaminated with small quantities of A, and the recognition of an actinomycin B was dropped. The homogeneity of actinomycin A was based on the constant optical rotation and absorption spectra obtained when preparations of the antibiotic were crystallized repeatedly from acetone and from ethyl acetate.

Further studies[4] revealed the fact that actinomycin is produced by different species of actinomycetes belonging largely to the genus *Streptomyces*. Since then, numerous investigators have reported the isolation of actinomycin or actinomycin-like substances from different cultures of *Streptomyces*.[5, 6, 7, 8]

A crystalline preparation, isolated by Lehr and Berger[9] and designated "Antibiotic X-45," exhibited activity against bacteria and fungi and toxicity in mice very similar to that of actinomycin A. The color, melting point, optical rotation, and absorption spectrum of this preparation were in good agreement with the published data for actinomycin A. However, the elementary analyses (carbon value) and the molecular weights of these preparations appeared to differ. Dalgliesh and Todd[10] re-examined "Antibiotic X-45" and designated it actinomycin B. While some discrepancies existed between the analytical values of the two preparations, there was a remarkable degree of coincidence in their chemical and physical properties and, in particular, in their infrared spectra.[11] Vigorous acid hydrolysis of actinomycin B gave five amino acids: sarcosine, D-valine, L-threonine, L-proline, and *N*-methyl-L-valine.[12] On hydrolyzing a preparation of actinomycin A, the same five amino acids were found. The conclusion was reached that the two were probably identical.

Soon after, Brockmann and Grubhofer[13, 14] isolated from a culture of *S. chrysomallus* another actinomycin which they designated as C. It appeared to be different from A and B on the basis of crystal form, melting point, solubility, color reaction in ethanolic alkali, infrared spectrum (between 1500 and 700 $cm.^{-1}$), analytical composition (especially the lower nitrogen content), and the nature of the amino acids present. Hydrolyzates of actinomycin C contained the six amino acids: D-valine, sarcosine, L-threonine, L-proline, *N*-methyl-L-valine, and D-alloisoleucine.

As a result of screening various cultures for new actinomycin preparations, Brockmann and Pfennig[15] and Brockmann *et al.*[16] reported the isolation of a crystalline product that differed from actinomycin C in certain physical and chemical properties and that gave, on hydrolysis, the same amino acids as actinomycin B. It was concluded that this antibiotic was very similar to B; nevertheless, it was labeled "actinomycin X." Shortly thereafter, Brockmann[17] and Brockmann and Gröne[18] reported the isolation of another actinomycin preparation, which was considered to be similar to actinomycin A but was given the designation "actinomycin I."

Beginning in 1953, a renewed search for actinomycin-producing strains was conducted in our laboratories, resulting in the isolation from cultures of *S. parvullus* of still another crystalline substance which was named "actinomycin D."[19]

In addition to the actinomycins listed above, the alphabetical designations AA, AC, J, and M have been employed by several investigators to name other preparations isolated by them.

Multiple Nature of the Actinomycins.—Although actinomycins A, B, and C had been obtained as crystalline substances and were considered to be homogeneous compounds by criteria such as constancy of melting point and optical rotation after repeated recrystallization, it was soon realized that actinomycin C was not a single chemical compound but a mixture of very similar substances.[14] This conclusion was based on the fact that actinomycin C, prepared at different times, exhibited different characteristics (e.g., solubility in absolute alcohol, analytical values). By means of countercurrent distribution and circular paper chromatography, it was possible to separate actinomycin C into three distinct components, which were designated C_1, C_2, and C_3.[15, 20, 21] Subsequently, by countercurrent distribution and partition chromatography on cellulose columns, Brockmann and Gröne[22] also demonstrated the presence of two other components, present in trace amounts, which they named C_{0a} and C_0. The chemical and physical properties of the various components are given in Table 1. Unfortunately, little biological information was presented.

Employing the same techniques of countercurrent distribution, circular paper chromatography, and cellulose column chromatography, Brockmann *et al.*[16, 18, 21, 22] separated actinomycin X into a number of components, designated X_{0a}, X_0, X_1, X_{1a}, X_2, X_3, and X_4. Only X_1 and X_2 were obtained in crystalline form, the latter representing 80–90 per cent of the complex. Brockmann and Pampus[23] reported briefly on the isolation of a crystalline fraction from X_0 which they named $X_{0\beta}$. The remaining components were present in trace quantities and have not been studied further (Table 1). It was also reported that $X_{0\beta}$ was four to five times less active against *Staphylococcus aureus* than X_1 and X_2.

Actinomycin I was also shown to be a mixture of several components, designated I_{0a}, I_0, I_1, I_2, and I_3. Only actinomycin I_0 and I_1 have been obtained in crystalline form, the latter making up more than 80 per cent of the complex.[22] No information was presented concerning their biological activity (Table 1).

Brockmann and Gröne[18] also examined samples of the actinomycin A studied by Waksman and Tishler[3] and the actinomycin B studied by Dalgliesh and Todd.[11, 12] They reported that actinomycins A and I were identical on the basis of their amino acid content, melting point, and behavior to circular paper chromatography.

TABLE 1

PROPERTIES OF PURE ACTINOMYCINS*

Actinomycin Complex	Actinomycin Component	Crystal Form	Melting Point† (°C.)	Specific Rotation‡	Specific Extinction§	Amino Acid Composition (Moles/Mole of Actinomycin Mol. Wt. 1,200)							Relative Percentage of Components in Actinomycin Complex
						L-Threonine	Sarcosine	L-Proline	D-Valine	N-CH$_3$-L-Valine	D-Allo-Isoleucine	Hydroxy Proline	
C	C_1	Bipyramids, prisms, or needles	241–243	$[\alpha]_D^{30} = -349° \pm 10°$	20.5	2	2	2	2	2	0	0	C_0, C_{0a} } 2.0 C_1, 18.1
	C_2	Same as C_1	237–239	$[\alpha]_D^{19} = -325° \pm 10°$	19.9	2	2	2	1	2	1	0	C_2, 45.7
	C_3	Same as C_1	232–235	$[\alpha]_D^{19} = -321° \pm 10°$	18.8	2	2	2	0	2	2	0	C_3, 34.4
X	$X_{0\beta}$	Yellow-red needles	245–247	$-261° \pm 10°$	17.1	+	+	+	+	+	0	+	X_{0a}, X_0 } 5.1
	X_1	Same as C_1	241–242	$[\alpha]_D^{30} = -309° \pm 10°$	17.3	2	2	2	2	2	0	0	X_1, 5.1 X_{1a}, trace
	X_2	Rhombic plates	244–246	$[\alpha]_D^{19} = -341° \pm 10°$	18.6	2	2	1	2	2	0	0	X_2, 88.6 X_3, 0.5 X_4, 0.5
I	I_0	Same as C_1	242–243	$[\alpha]_D^{30} = -314° \pm 10°$	20.5	+	+	+	+	+	0	0	I_{0a}, 0.5 I_0, 7.3
	I_1	Same as C_1	240–242	$[\alpha]_D^{30} = -353° \pm 10°$	20.7	+	+	+	+	+	0	0	I_1, 86.5 I_2, 5.6 I_3, 0.5

* See Brockman and Gröne, *Naturwissenschaften*, **41**, 65, 1954, and *Chem. Ber.*, **87**, 1036, 1954; Brockman and Pampus, *Angew. Chem.*, **67**, 519, 1955.
† Kofler-Block.
‡ In methanol
§ At 445 mμ in methanol.

Actinomycins B and X behaved exactly alike on chromatograms, and the agreement in their melting points, amino acid contents, and other properties led to the conclusion that they were also identical. Subsequently, two crystalline components (named B_1 and B_2) were isolated from the B mixture and found to be identical with X_1 and X_2.[22]

Brockmann and Gröne[22] thus reported a total of 17 fractions from the actinomycins C, X, and I. Thirteen of these components were found to be different, namely, C_{0a}, C_0, C_1, C_2, C_3, X_{0a}, X_0, X_{1a}, X_1, X_2, X_3, X_4, and I_0; three were similar, namely, $C_{0a} = I_{0a}$, $C_1 = I_1$, and $C_2 = I_2$; it was also suggested that C_3 could be identical with I_3, although this was not certain.

The confusion in the actinomycin nomenclature had now attained the highest degree. Not only were actinomycins A, B, C, D, X, etc., reported, but each of these was found to be a mixture of closely related chemical components; it further appeared that each component was different from most of the others.

To ascertain whether such a multiplicity of actinomycins actually existed and to attempt a rationalization of the highly complicated actinomycin nomenclature, Vining and Waksman[24] and Gregory *et al.*[25] used paper chromatography for the separation of the various crystalline actinomycin preparations. They demonstrated that A, B, X, and C were mixtures of a number of components, whereas D was essentially a homogeneous substance. They also showed that the C_1 of actinomycin C and the D component were present in the A, B, and X preparations and that B and X actinomycins consisted essentially of the same components in slightly different proportions. Actinomycin A was very similar to B and X, whereas two of the components of C, namely, C_2 and C_3, were unique and were not found in any of the other preparations.

Roussos and Vining[26] subsequently corroborated and extended these initial findings. By means of circular paper chromatography and partition chromatography on powdered cellulose columns, they separated seven pure components from actinomycins A, B, and D. An examination of their chemical properties (Table 2) revealed that the actinomycin component A_I is equivalent to B_I, that $A_{IV} = B_{IV} = D_{IV}$, and that $A_V = B_V$. A study of their biological properties fully confirmed these conclusions.[27] On the basis of these results, Roussos and Vining suggested that the naturally produced actinomycins A, B, X, and D are made up of the same components and that the differences observed may be attributed to the relative amounts of each produced by the respective *Streptomyces* species.

Composition of Actinomycin A.—An examination of the various samples of actinomycin A isolated in our earlier studies (1940–42) and of the more recent preparations, obtained from the same culture of *S. antibioticus*, revealed the fact that during the initial isolation and purification of actinomycin A on alumina[3] one of its components (A_I) was apparently lost. Such a sample of actinomycin A was sent to Brockmann for his examination. On the basis of paper chromatograms, Brockmann and Gröne[18] considered it to be a homogeneous substance and at first claimed that it was similar to, if not identical with, actinomycin I; later, however, they concluded that it was similar to the I_1 component.[22]

We have also examined the same A material but concluded that it was a mixture rather than a homogeneous entity. The components present corresponded in Rf value with those of B, though the relative amounts of each differed in the two

TABLE 2

PROPERTIES OF PURE ACTINOMYCINS*

Actinomycin Complex	Actinomycin Component	Crystal Form	Melting Point† (° C.)	Specific Rotation‡	Specific Extinction	Amino Acid Composition (Moles/Mole of Actinomycin of Mol. Wt. 1,200)					Relative Percentage of Components in Complex
						L-Threonine	Sarcosine	L-Proline	D-Valine	N-CH$_3$-L-Valine	
A	A_I	Plates	237–238	...	18.0	2	2	1	2	2	A_I, 6.6 A_{II}, 2.9
	A_{IV}	Prisms	235–236	−261°	19.2	2	2	2	2	2	A_{III}, tr. A_{IV}, 66.7
	A_V	Fine Needles	245–246.5	−323°	19.6	2	2	1	2	2	A_V, 23.8 A_{VI}, tr.
B	B_I	Plates	237.5–238	−235°	17.8	2	2	1	2	2	B_I, 9.6 B_{II}, tr.
	B_{IV}	Prisms	235.5–236.5	−268°	18.8	2	2	2	2	2	B_{III}, tr. B_{IV}, 28.1
	B_V	Fine Needles	246–246.5	−320	19.2	2	2	1	2	2	B_V, 59.3 B_{VI}, 3.1
D	D_{IV}	Prisms	235.5–236.5	−262°	19.6	2	2	2	2	2	D_{IV}, 100 D_I, D_{II}, D_{III}, D_V in trace amounts

* See Roussos and Vining, *J. Chem. Soc.*, p. 2469, 1956.
† Kofler hot plate.
‡ $[\alpha]_D^{23}$ (C, 0.25 in 95 per cent EtOH).

samples. Moreover, an A_1 component was lacking.[24–26] Since Brockmann and co-workers had reported that there were differences between I and X(B) in their behavior to paper chromatography in certain solvent systems as well as in the properties of the isolated pure components of each complex, the two sets of results appeared to be in conflict. It was concluded by Roussos and Vining[26] that Brockmann's earlier paper chromatographic evidence was probably in error and

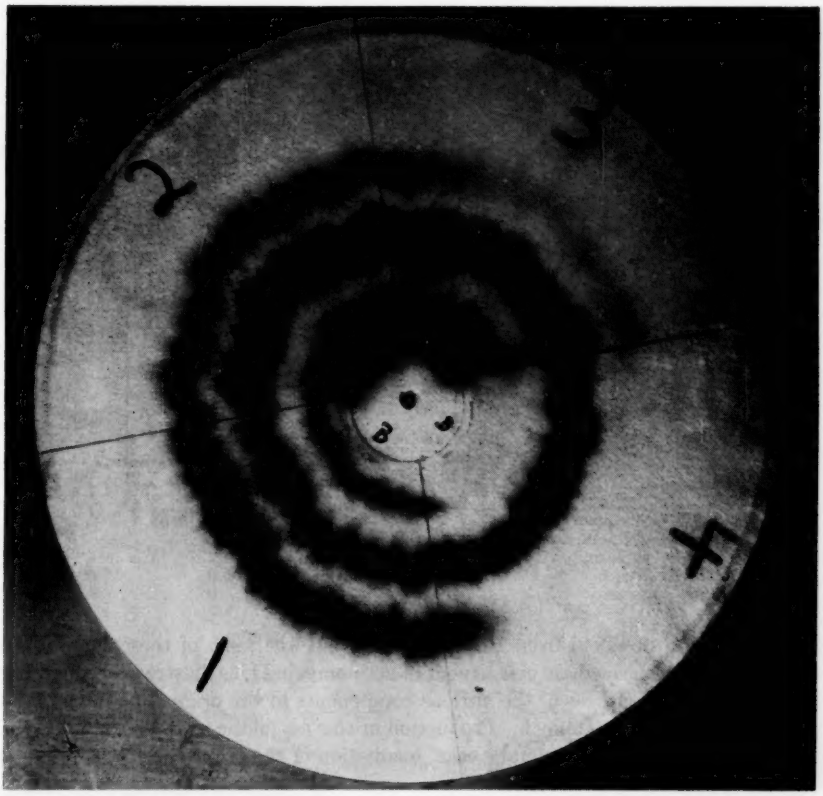


FIG. 1.—Circular paper chromatographs of actinomycin A, B, and D preparations. The system was composed of equal parts of a 10 per cent aqueous solution of sodium-*ortho*-cresotinate, and a mixture of ethyl acetate and *n*-dibutyl ether (2:1). *1*, actinomycin B; *2*, actinomycin A produced in 1948; *3*, actinomycin A produced in 1940; *4*, actinomycin D.

that, in fact, actinomycin I was different from A. The evidence for a close relationship between I and C seemed more plausible.[22]

In 1953, in order to carry out additional comparative studies with actinomycin A, we prepared fresh lots of this actinomycin employing the original culture of *s. antibioticus*, from which actinomycin A was first isolated in 1940. We found that the material produced could be considered as a B-type complex. While these results were quite unexpected, it was believed possible that the difference in the

process of cultivation of the organism employed in 1940 (surface process) and in 1953 (shaken process) might influence the composition of the actinomycin thus produced.

Since some questions have arisen concerning the composition of the actinomycin A complex, due, on the one hand, to the loss of component A_I through purification[3] and, on the other, to the production of a B-type complex[25] in 1955, we decided to enlarge upon these investigations to resolve these discrepancies.

It has been possible to obtain from several different laboratories samples of the actinomycin A produced in our laboratory in 1940 and in 1948, and placed at that time at the disposal of various investigators in those laboratories. By means of circular paper chromatography, we were now able to show that actinomycin A preparations consisted of several components. By the use of different solvent systems, these components were found to be identical with those present in a sample of

TABLE 3

RELATIVE PERCENTAGES OF VARIOUS COMPONENTS OF ACTINOMYCIN A PREPARATIONS

COMPONENT	RELATIVE PERCENTAGE OF COMPONENTS	
	Actinomycin A Produced in 1940	Actinomycin A Produced in 1948
A_I	7.9	7.0
A_{IV}	84.6	71.7
A_V	7.5	24.1

TABLE 4

INFLUENCE OF NITROGEN SOURCE ON COMPOSITION OF ACTINOMYCIN COMPLEX

ORGANISM	NITROGEN SOURCE	RELATIVE PERCENTAGE OF COMPONENTS						
		I	II	III	IV	V	VI	VII
S. antibioticus	L-glutamic acid	6.2	2.1	2.8	80.4	8.4	...	..
S. antibioticus	L-threonine	7.1	2.4	2.9	29.2	58.4	...	..
S. antibioticus	L-glutamic acid plus sarcosine	7.1	24.6	35.6	27.2	5.5	...	..
S. chrysomallus	KNO_3	..	...	..	10.0		60.0	40.0
S chrysomallus	Glycine	..	...	..	7.0		43.2	49.8

actinomycin B obtained from Hoffmann-LaRoche, Inc.; one of these components was identical with the main constituent of actinomycin D, as illustrated in Figure 1. The relative percentages of the various components in the original actinomycin A complex are given in Table 3. Production of this antibiotic was carried out under static conditions during several weeks' incubation of the culture, a procedure used in 1940.

Recent studies with another strain of *S. antibioticus* grown under both static and shaken conditions have revealed that, with increased time of incubation, there occurs in the actinomycin complex a continuous rise in the relative percentage of component A_{IV} to 70–80 per cent and even more, and a concomitant drop of A_I to 5 per cent and of A_V to 10–20 per cent. Purification of such actinomycin A preparations was accompanied by a selective loss of A_I (adherence to impurities on the alumina column, discarding certain fractions of the eluate), which thus gradually resulted in an actinomycin A lacking A_I. This conceivably occurred during the purification procedure employed by Waksman and Tishler[3]; the actinomycin A thus obtained in crystalline form consisted essentially of A_{IV} and A_V components.

The explanation for the production of a B-type complex by *S. antibioticus* in

1953 resides solely in the poor yields of the actinomycin. This culture, regardless of the medium used, was now unable to form more than a microgram or two of actinomycin per milliliter of broth. Recently it was found that cultures of *S. antibioticus* are able to form a B-type complex during the early stages of actinomycin production (0–30 $\mu g/m_1$) and later an A-type complex (30–150 μ_2/ml), when grown on complex as well as on chemically defined media.[28, 29] The actinomycin extracted from a broth during the early stages of growth or when the antibiotic yield was extremely low was a B-type mixture.

Effect of Age of Culture and Composition of Medium.—Further studies of the course of biosynthesis of actinomycin yielded much information that has a bearing

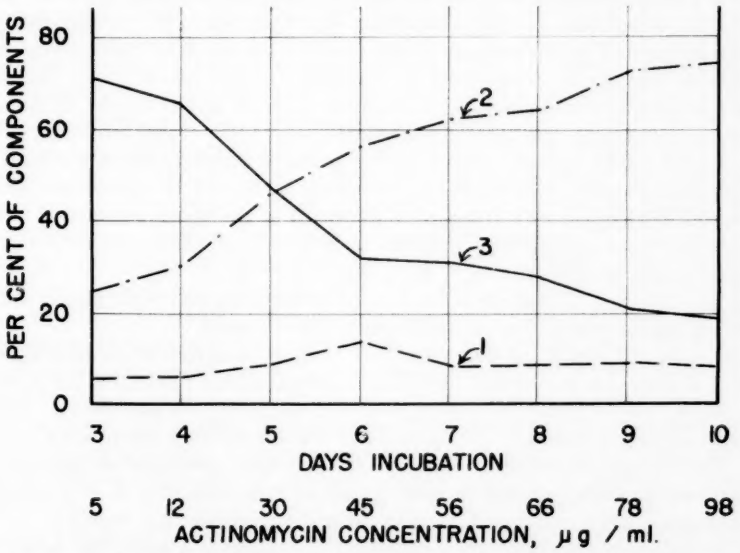


FIG. 2.—Relative percentages of the components in an actinomycin preparation produced by *S. antibioticus* 3720, growing on a galactose–glutamic acid–mineral salts medium. *1*, component A_I; *2*, component A_{IV}; *3*, component A_V.

upon the question of the nomenclature of the actinomycins. This information may be summarized briefly as follows:

During the course of actinomycin production in various complex and in chemically defined media by several strains of *S. antibioticus*, the proportion of the various components in the actinomycin preparations varied from day to day, so that a single component might represent 30 per cent of the actinomycin at one time and 70 per cent at another (Fig. 2). This was also true of other *Streptomyces* species producing actinomycin.[29]

The nature of the actinomycin formed by the various organisms also depended to a considerable extent on the nitrogen supply in the medium.[29] When threonine was present, *S. antibioticus* produced only the B-type complex, but when glutamic acid was supplied as the sole nitrogen source, the B type was first produced and then the A type. *Streptomyces* species 3723 (Streptomyces X-45 of Lehr and Berger)

produced a B-type actinomycin on many complex media, but, when supplied with glutamic acid, glycine, or KNO_3 in a chemically defined medium, the A type was formed. The actinomycin C producer, *S. chrysomallus*, gave a C-type complex which contained C_2 as the major component when KNO_3 was the source of nitrogen, but C_3 was the major constituent when glycine was the sole nitrogen source.

Discussion.—On the basis of the various chemical, biological, and microbiological studies presented in this paper, it is proposed that a revision of the existing actinomycin nomenclature be made.

Since a single *Streptomyces* culture is capable of producing mixtures of actinomycins with widely varying proportions of components, depending almost as much on the nutrient medium used and the age at which the culture is harvested as on the strain of organism, the use of symbols to designate either the number, the type, or the proportion of components present is certain to result in hopeless confusion. Moreover, the precise composition of the mixture would not be clearly indicated.

We now know that actinomycin complexes A, B, X, and D consist of the same chemical components but differ in the relative proportions of each present.[26] Actinomycin A (1940) actually represents the primary complex, which possesses the different components found in the actinomycins B, X, and D. Moreover, actinomycin C_1 is identical with component IV found in the above complexes. These results have been confirmed and extended by Brockmann‡, who has shown by circular paper chromatography that $A_I = B_I = X_{0\beta}$; $A_{IV} = B_{IV} = D_{IV} = C_1 = I_1 = X_1$; and $A_V = B_V = X_2$. Actinomycin C_2 and C_3 remain the only other clearly defined chemical entities which have been sufficiently characterized.

Corbaz *et al.*[30] concluded that there were three actinomycin types, namely, C, I, and X. They also stated that they clearly recognized the priority of the A and B complexes; however, they promptly proceeded to ignore such precedence. Brockmann[31] also recognizes only the existence of the actinomycin complexes C, I, and X. On the basis of the data obtained by Brockmann and reported here, as well as our own findings, it would be more proper to state that A = B = X and that D represents only a component (A_{IV}) found in the actinomycin A complex.

The relationship of actinomycin complex I to complexes A and C has never been clearly settled. Published paper chromatograms of I are not sufficiently well defined, so that it is impossible to decide what its components are related to. Brockmann has indicated, at one time or another, that I = A; I_1 = A; $C_{0a} = I_{0a}$, $C_1 = I_1$, $C_2 = I_2$; $C_1 = I_1 = A_{IV}$, and, in a recent publication,[31] I = D. If I is nearly homogeneous but possesses additional components in small concentrations, it may be similar to an A complex harvested after prolonged incubation of the culture (Table 3); or it may represent an A complex which has lost certain components through purification, or it may be similar to actinomycin D. It may also be a C complex, with C_1 as major component. In this case it should contain D-alloisoleucine. Data published on the amino acid content of the I complex and the pure components I_0 and I_1 indicate that it is absent, but if I_2 and I_3 are equivalent to C_2 and C_3 respectively and represent only about 6 per cent of the complex, it is not unlikely that its presence may have been overlooked on the chromatogram. In view of the results of Brockmann and Gröne[22] quoted above, it appears possible that I may bear the same relationship to C as A does to B and X.

The actinomycin preparations AA, AC,[32, 33] M,[34] and J[7, 35, 36] have been inade-

quately studied, and it is not entirely certain whether they represent new or previously described actinomycin complexes or components. For example, actinomycin AA and AC are fractions obtained by Sarlet through alumina chromatography of an actinomycin preparation. The amino acid content of the material was identical with that of actinomycin C; the amino acid composition of the fractions suggested that AA = A_{IV} (C_1) and AC = C_2. Actinomycin M was examined in our laboratory and found to be of the B type. Until the nature of these preparations is more completely known, we propose that their designations be abolished.

Trace components designated as C_{0a}, X_{0a}, X_4, etc.,[22] may represent either actinomycin components or degradation products derived therefrom. The amount of these substances isolated in crystalline form has been negligible, and consequently little or no information is available concerning their chemical and biological properties. Until more data are published, we propose that such substances remain unclassified; the present practice of giving a designation to these poorly defined fractions has resulted in a rash of meaningless letters and numbers which contribute nothing but confusion to the actinomycin nomenclature.

The new biosynthetic actinomycins, designated E and F by Schmidt-Kastner,[37] and similar preparations we have obtained in our own laboratory may represent a unique group of actinomycins which have been produced through special conditions of nutrition. Whether they should be given new designations, e.g., E and F, or represent members of an actinomycin type normally produced in trace amounts, e.g., with sarcosine *S. antibioticus* produces an A complex containing up to 60 per cent actinomycin A_{II} and A_{III}, remains to be decided on the basis of more complete chemical and biological characterization. This is brought out in Table 4.

Conclusions.—On the basis of the facts presented above, we therefore propose the following:

1. The term "actinomycin" should be restricted in use to chemically pure substances. Where a product is known to consist of a mixture, it should be referred to as an "actinomycin complex" or "actinomycin mixture," to indicate chemical inhomogeneity.

2. The present system of classification based on the combination of components present in varying amounts, e.g., A, B, X, C, etc., be abandoned. Also the use of letters, subscripts, and other confusing symbols be done away with. Since identical actinomycins can be found in different complexes, the use of any symbol indicating the complex of origin should be avoided. The pure actinomycin components should be renamed, but, in order to avoid superimposing a completely new set of symbols, only the Roman subscripts already employed shall be used:

A_I, B_I, $X_{0\beta}$	= Actinomycin I,
A_{II}, B_{II}	= Actinomycin II,
A_{III}, B_{III}	= Actinomycin III,
A_{IV}, B_{IV}, D_{IV}, C_1, I_1, X_1	= Actinomycin IV,
A_V, B_V, X_2	= Actinomycin V,
C_2	= Actinomycin VI,
C_3	= Actinomycin VII.

Other minor components of different complexes and the new biosynthetic actino-

mycins might all be given a similar distinguishing symbol as soon as their homogeneity and difference from already named types is established.

3. The naturally produced actinomycin complexes will thus be made up of the various actinomycins in different proportions. What was known as "actinomycin A" will simply be an "actinomycin mixture" consisting of actinomycins I–V, and "actinomycin C" will consist of actinomycins IV, VI and VII.

* This study was supported by a research grant from the United States Public Health Service and by funds supplied by Rutgers Research and Educational Foundation.

† Now located at Prairie Regional Research Laboratory, Saskatoon, Saskatchewan, Canada.

‡ Personal communication.

[1] S. A. Waksman and H. B. Woodruff, *Proc. Soc. Exptl. Biol. Med.*, **45**, 609, 1940.

[2] S. A. Waksman, H. Robinson, H. J. Metzger, and H. B. Woodruff, *Proc. Soc. Exptl. Biol. Med.*, **47**, 261, 1941.

[3] S. A. Waksman and M. Tishler, *J. Biol. Chem.*, **142**, 519, 1942.

[4] S. A. Waksman, W. B. Geiger, and D. M. Reynolds, these PROCEEDINGS, **32**, 117, 1946.

[5] M. Welsch, *Comp. rend. soc. biol.*, **140**, 1157, 1946.

[6] P. C. Trussell and E. M. Richardson, *Can. J. Research*, **26c**, 27, 1948.

[7] H. Umezawa, S. Hayano, T. Takeuchi, and Y. Mizuhara, *Japan Med. J.*, **1**, 100, 1948.

[8] W. Kocholaty, W. R. Junowicz-Kocholaty, and A. Kelner, *Arch. Biochem.*, **17**, 181, 1948.

[9] H. Lehr and J. Berger, *Arch. Biochem.*, **23**, 503, 1949.

[10] C. E. Dalgliesh and A. R. Todd, *Intern. Congr. Biochem. Abstr. Comm. 1st Congr., Cambridge, England*, p. 246, 1949.

[11] C. E. Dalgliesh and A. R. Todd, *Nature*, **164**, 830, 1949.

[12] C. E. Dalgliesh, A. W. Johnson, A. R. Todd, and L. C. Vining, *J. Chem. Soc.*, p. 2946, 1950.

[13] H. Brockmann and N. Grubhofer, *Naturwissenschaften*, **36**, 376, 1949.

[14] *Ibid.*, **37**, 494, 1950.

[15] H. Brockmann and N. Pfennig, *Naturwissenschaften*, **39**, 429, 1952.

[16] H. Brockmann, H. Linge, and H. Gröne, *Naturwissenschaften*, **40**, 224, 1953.

[17] H. Brockmann, *Angew. Chem.*, **66**, 1, 1954.

[18] H. Brockmann and H. Gröne, *Naturwissenschaften*, **41**, 65, 1954.

[19] R. A. Manaker, F. J. Gregory, L. C. Vining, and S. A. Waksman, *Antibiotics Ann.*, p. 853, 1954/1955.

[20] H. Brockmann, N. Grubhofer, W. Kass, and H. Kalbe, *Chem. Ber.*, **84**, 260, 1951.

[21] H. Brockmann and H. Gröne, *Naturwissenschaften*, **40**, 222, 1953.

[22] H. Brockmann and H. Gröne, *Chem. Ber.*, **87**, 1036, 1954.

[23] H. Brockmann and G. Pampus, *Angew. Chem.*, **67**, 519, 1955.

[24] L. C. Vining and S. A. Waksman, *Science*, **120**, 389, 1954.

[25] F. J. Gregory, L. C. Vining, and S. A. Waksman, *Antibiotics and Chemotherapy*, **5**, 409, 1955.

[26] G. G. Roussos and L. C. Vining, *J. Chem. Soc.*, p. 2469, 1956.

[27] L. H. Pugh, E. Katz, and S. A. Waksman, *J. Bacteriol.*, **72**, 660, 1956.

[28] W. A. Goss, E. Katz, and S. A. Waksman, these PROCEEDINGS, **42**, 10, 1956.

[29] W. A. Goss and E. Katz, *Appl. Microbiol.*, **5**, 95, 1957.

[30] R. Corbaz, L. Ettlinger, W. Keller-Schierlein, and K. Zähner, *Arch. Mikrobiol.*, **26**, 182, 1957.

[31] H. Brockmann, *Giorn. ital. chemioterap.*, **3**, 491, 1956.

[32] H. Sarlet, *Enzymologia*, **14**, 49, 1950.

[33] H. Sarlet, *Biochim. et Biophys. Acta*, **13**, 143, 1954.

[34] V. Monesi, V. Veronesi, and R. Craveri, Boll. Soc. ital. Ematol., **2**, 243, 1954.

[35] H. Umezawa, K. Maeda, and Y. Okami, *J. Antibiotics* (Japan), **4**, 335, 1951.

[36] A. Nishibori, *J. Antibiotics*, Ser. A, **9**, 31, 1956.

[37] G. Schmidt-Kastner, *Naturwissenschaften*, **43**, 131, 1956.

*ON THE NATURE OF FREE RADICALS DETECTED BY PARAMAGNETIC RESONANCE IN BIOLOGICAL SUBSTANCES**

By Ichiro Miyagawa, and Walter Gordy,

DEPARTMENT OF PHYSICS

Norimitsu Watabe, and Karl M. Wilbur

DEPARTMENT OF ZOÖLOGY,

DUKE UNIVERSITY, DURHAM, NORTH CAROLINA

Communicated by James Franck, April 15, 1958

Observations of weak, relatively sharp electron-spin resonance signals in a variety of lyophilized biological materials have been reported by Commoner, Townsend, and Pake.[1] Studies at Duke University[2] have shown that similar signals can be produced in numerous organic chemicals, including biological substances, by X-irradiation in oxygen or by the admission of oxygen to samples which had previously been irradiated in a vacuum. These signals produced by oxygen addition have no nuclear hyperfine structure but have a small asymmetry in the g factor of the type expected for an electron localized on a bound oxygen molecule. The signals thus produced are long-lived, usually much more so than the signals formed by irradiation without oxygen.

Because similar, long-lived signals can be produced by addition of oxygen to irradiated biological materials, it seems possible that signals like those observed by Commoner, Townsend, and Pake[1] in unirradiated biological substances might arise from peroxide radicals, ROO, or from semibonded, trapped molecular oxygen. Indeed, this possibility had been suggested earlier by Shields, Ard, and Gordy,[3] who observed similar signals along with signals from paramagnetic metallic ions in plant materials which had not been lyophilized or irradiated. Peroxide radicals might be built up to detectable proportions by combination of oxygen with radicals which themselves never reach detectable proportions or which have resonances too broad for detection with the usual spectrometers. Detectable paramagnetic oxygen may also be trapped by certain substances or charged groups which are not themselves radicals and do not have an unpaired spin. Resonance of gaseous, untrapped oxygen is difficult to detect under atmospheric conditions because the signals are spread out by interaction of the electronic spin with the molecular rotation and by molecular collisions. Gaseous oxygen would not be observable in our experiments.

In the present experiments we have sought to determine whether the signals of the type observed by Commoner, Townsend, and Pake[1] arise from peroxide radicals or trapped oxygen. We have been able to demonstrate that such signals can indeed be produced by the admission of molecular oxygen to lyophilized—but unirradiated—biological materials such as rat spleen, liver, kidney, or heart. The results strongly suggest that the greater part, if not all, of the natural electron-spin resonance signal strength in such materials results from absorbed oxygen, probably weakly bonded to some as yet unknown element or group in the material.

For most measurements, tissue was removed from adult male Sprague-Dawley rats immediately after cervical dislocation and severing the jugular veins, sliced quickly by hand on a glass surface cooled to 0°, and put into liquid nitrogen. This

process took about 5 minutes. In most experiments the tissue was carefully ground in a mortar in liquid nitrogen and lyophilized in vacuum for 5 hours. Some specimens were lyophilized without grinding. In some experiments the sample was lyophilized at 0° and never cooled below this temperature. Oxygen and nitrogen were dried by P_2O_5 for 1 hour or more but were not purified. The measurement of the paramagnetic resonance absorption was made at 23 kMc/sec at room temperature. The usual magnetic modulation spectrometer was employed with an Esterline-Angus pen-and-ink recorder which displayed the first derivative of the actual absorption curves.

The samples were lyophilized and observed first in vacuum. Later dry air, oxygen, nitrogen, carbon monoxide, or carbon dioxide at 1 atmosphere pressure was admitted to the sample, and its paramagnetic resonances were measured at various

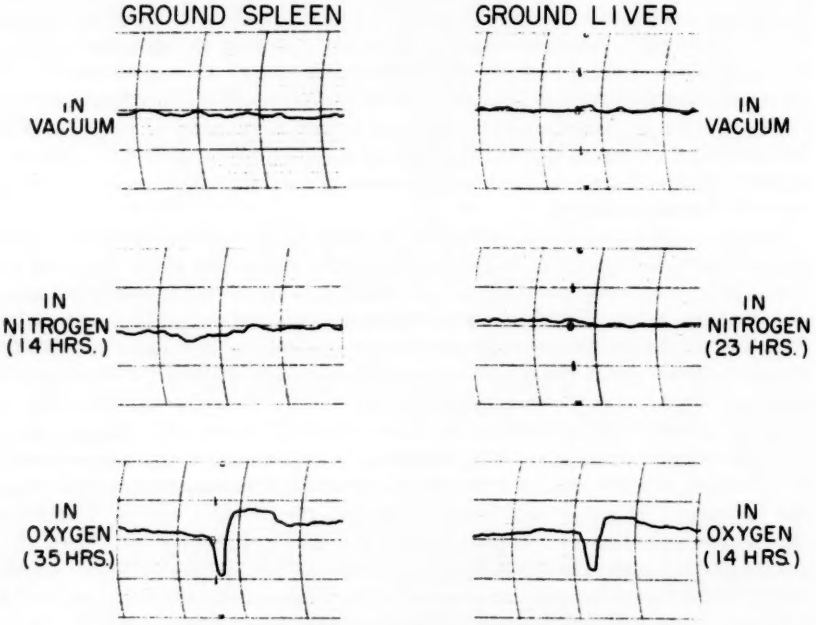


FIG. 1.—Electron-spin resonance curves obtained at 23,000 Mc of lyophilized, ground samples of rat tissue showing detectable signals in oxygen but not in nitrogen or vacuum. The g factor for the observed resonance is 2.00.

intervals afterward. No signal, or only a very weak signal, was observed until oxygen was admitted. Typical results are shown in Figures 1 and 2. A few hours after air or oxygen was admitted—and for some samples only a few minutes afterward—a sizable resonance signal was detected. The effects of oxygen or dry air are contrasted with the null effects of nitrogen in Figures 1 and 2.

The signals produced by the admission of oxygen or air to the lyophilized samples are evidently due to molecular oxygen weakly bonded to something in the lyophilized sample. That the signal can again be reduced considerably by thoroughly pumping off the oxygen is evidence for the weakness of the bonds. This effect is

demonstrated in Figure 2. When oxygen is admitted a second time, the signal returns, although possibly not quite to its original intensity. In the sliced, unground samples it is not possible to produce such large variations in the signals by pumping the samples, presumably because of the slow diffusion rate of the oxygen into and out of the samples.

The shape of the paramagnetic resonance curves of lyophilized tissues allowed to remain in oxygen is like that obtained when the sample is X-irradiated and allowed to remain in oxygen. This shape is evident in Figure 3, where a particularly strong

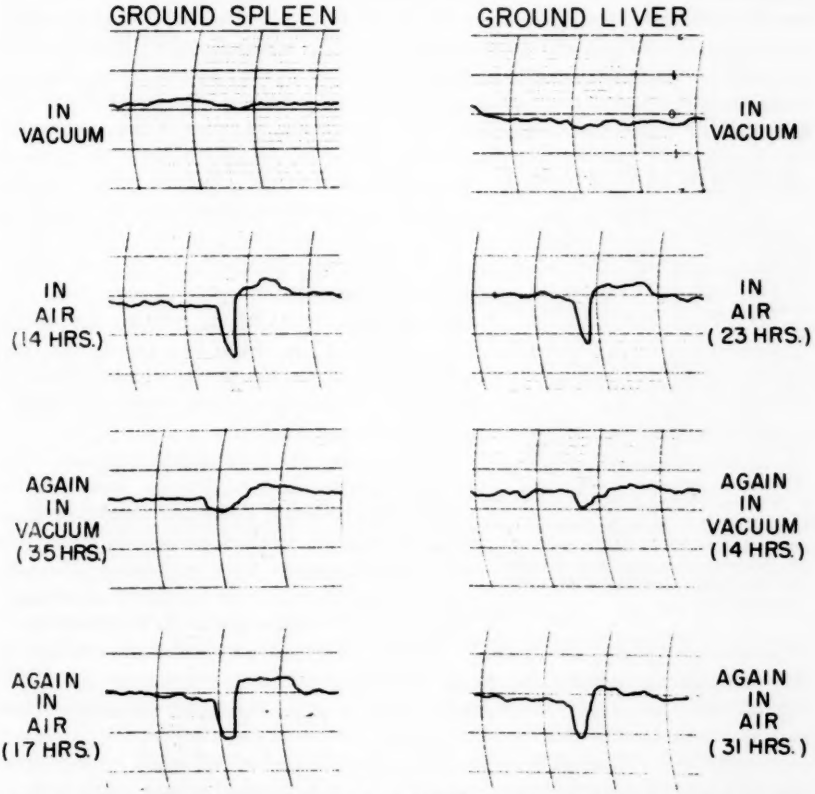


FIG. 2.—Electron-spin resonance curves at 23,000 Mc, showing that signals produced by admission of air can be reduced by pumping away the air and again increased by introducing air. The effects are due to oxygen in the air.

signal was obtained for a sliced sample. The shape of the resonance is asymmetric, with the weak shoulder on the high-field side. This shape is the one expected for polyoriented radicals having an axially symmetric, slightly anisotropic g factor. The weak shoulder arises from radicals with their symmetry axis approximately parallel to the applied field, and the stronger peak from those with the axis approximately perpendicular to the field. In the polyoriented samples the perpendicular orientation has the greater statistical weight. At the frequency of 9,000 Mc

employed by Commoner, Townsend, and Pake,[1] the shoulder is not noticeably separated from the stronger peak, and even at 23,000 Mc it is not very evident when the signals are very weak, as in Figures 1 and 2.

We found that the signal intensity obtainable from a given tissue varies from rat to rat. The average signal strength of many samples of the same tissue was compared with the average value for many samples of tissues of other types treated in the same manner. Using this method, we found that the signal intensities are generally in this order: spleen $\geqslant$ liver $>$ kidney $\geqslant$ heart.

One might ask why signals are not observed from oxygen trapped in the cell before the animal is killed. An answer is provided by the experiments, which show that the oxygen which gives the resonance can be pumped away. Such oxygen originally in the tissue would be greatly reduced by the pumping required for lyophilizing the samples, especially in the ground samples. Also, in the living cell such radicals may never reach detectable proportions because of the continuous reactions which go on to complete the process of oxidation in the fluid cells. Of course, oxygen radicals or radicals of other types produced in the living animal may be detectable with a spectrometer of higher sensitivity. Our experiments do not preclude the existence of detectable free radicals produced directly within the living animal. What they seem to prove is that the primary signal strength in the tissues observed here is due to oxygen which enters the tissues after the animal is killed.

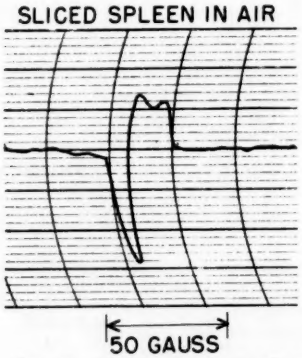


FIG. 3.—Strong signal, showing shape and width of first derivative of the resonance curve at 23,000 Mc for sliced, lyophilized spleen observed in air.

The fact that the natural paramagnetic resonances observed in animal tissue seem to arise merely from trapped molecular oxygen or from peroxide radicals does not necessarily mean that the observed effects have no biological significance or that there are not radicals of other kinds which may be intermediaries in biochemical reactions, as proposed by Michaelis.[4] However, the present experiments cause doubt that such radicals are often, if ever, in sufficient proportions to be directly detectable through their paramagnetic resonance. It is entirely reasonable that organic free radicals are the agents which are trapping the oxygen in these tissues, but this is not necessarily true. Molecular oxygen is itself paramagnetic and might remain paramagnetic while loosely held by electrostatic forces to charged groups which have an even number of electrons and are not themselves paramagnetic. Because most proteins and other organic substances for which the oxygen effect has been observed require prior irradiation for observation of the signal, it seems likely that either a free radical or some other active agent such as is produced by ionizing irradiation is necessary to trap the oxygen. Thus, in the unirradiated tissues for which the oxygen signals have been observed, there must be some active substance, perhaps a free radical or a charged group, which traps the oxygen. The trapping agents may be free radicals which have resonances too broad for detection until combination with oxygen takes place.

It seems reasonable that the active substance which traps the oxygen in these experiments may be the one which captures the oxygen molecule to start the process of oxygen consumption in the living cell. An experiment which we made indicates that the active agent which traps the oxygen is neutralized by water. If so, the agent must be maintained in small equilibrium concentrations with water and other chemicals in the living animal and, probably, it decays after the animal is killed. The experiment is this. Lyophilized tissues were put into a small quantity of water and refrigerated at 0° for several hours. They were then put into liquid nitrogen and were lyophilized in the usual way. When exposed to oxygen, these samples gave much weaker signals than did the samples not treated with water. For this reason our usual experiments were designed to dehydrate the sample as rapidly as possible after the animal was killed, on the assumption that water in the tissue neutralizes the oxygen-trapping agent.

We do not mean to imply in the above paragraph that only living processes can produce an active agent capable of trapping molecular oxygen. Irradiation can certainly produce such agents, as is proved by other experiments cited. Excessive heat and possibly decay of the animal tissues after death might do so. Shields, Ard, and Gordy[3] found that signals like those from trapped oxygen are sometimes strong in certain decaying plants. However, these plant substances were relatively dry and had been exposed to the sun's rays. It is possible that the active element in them was produced by solar radiations. Also it seems possible that the signals built up in etiolated barley leaves by hours of irradiation with a fluorescent light by Commoner, Townsend, and Pake[1] result from the formation of an active agent which traps oxygen. In later experiments radicals of another kind—radicals of much shorter lifetimes or molecules in triplet states—have been detected in chloroplasts exposed to light.[5, 6]

* This research was supported in part by the Office of Ordnance Research, Department of the Army, by the Office of Naval Research, and by the Atomic Energy Commission.

[1] B. Commoner, J. Townsend, and G. Pake, *Nature*, **174,** 689, 1954.

[2] W. Gordy, W. B. Ard, and H. Shields, these PROCEEDINGS, **41,** 983, 1955, and later unpublished results.

[3] H. Shields, W. B. Ard, and W. Gordy, *Nature*, **177,** 984, 1956.

[4] L. Michaelis, in *Currents in Biochemical Research*, ed. D. E. Green (New York: Interscience, 1946).

[5] B. Commoner, J. J. Heise, and J. Townsend, these PROCEEDINGS, **42,** 710, 1956.

[6] P. B. Sogo, N. G. Pon, and M. Calvin, these PROCEEDINGS, **43,** 387, 1957.

ADDENDUM TO SPIN, STATISTICS, AND THE TCP THEOREM

BY JULIAN SCHWINGER

HARVARD UNIVERSITY

In the preceding paper[1] the single physical requirement of the existence of a vacuum state was used to exclude Fermi-Dirac fields with integer spin, and Bose-Einstein fields of half-integer spin. While it is desirable to unify the treatment of all fields, the avoidance of explicit reference to commutation properties cannot be completely maintained. Indeed, the fundamental objection to fields of the type

ψ_{int} is on the kinematical, rather than the dynamical level (which was quite well appreciated while writing "S, S, TCP.").

The application of the stationary action principle yields an expression for the generator of field variations on a given spacelike surface,

$$G = \int d\sigma_\mu \chi A^\mu \, {}^1/_2\delta\chi = \int d\sigma \chi A^0 \, {}^1/_2\delta\chi,$$

the interpretation[2] of which,

$$\frac{1}{i}[\chi, G] = {}^1/_2\delta\chi,$$

is complicated only by the generally singular nature of the matrix A^0, which property, directly or indirectly, decreases the number of field components that appear explicitly in G and thus are subject to independent variation. For F.D. fields, where the field variations are anticommutative with the field, the commutation relations are contained in

$$\int d\sigma' \{\psi(x), \psi(x')\} \alpha^0 \delta_\psi(x') = \delta\psi(x),$$

where $\alpha^0 = -iA^0$ is a real, symmetrical matrix. The curly bracket, representing the symmetrical product of the Hermitian field operators, is a positive-definite structure, which demands the same characteristic for the non-singular submatrix of α^0 that refers to the independent field components. Hence there is a contradiction, for ψ_{int} fields, between this positiveness requirement on α^0 and the necessary existence of a transformation that converts α^0 into $-\alpha^0$.

We shall also add a comment on the structure of the matrices in the Lagrange function, specifically, of the fundamental Hermitian matrix B that enters in the part of $\mathfrak{H}$ associated with the individual fields,

$$\mathfrak{H}_0 = {}^1/_2 \chi B \chi.$$

The matrix B is symmetrical and real for B.E. fields, antisymmetrical and imaginary for F.D. fields. The invariance of $\mathfrak{H}_0$ under proper, orthochronous Lorentz transformations demands that B commute with the antisymmetrical spin matrices S_{kl}, and anticommute with the symmetrical matrices S_{ok}. This property is possessed by the space-reflection matrix,

$$R_s = R_1 R_2 R_3,$$

and thus

$$B = R_s I,$$

where I commutes with all $S_{\mu\nu}$. There are two classes of such matrices, distinguished by commutativity, or anticommutativity with R_s. According to the statement of the geometrical properties of reflections and rotations,

$$R_k R_l = e^{\pi i S_{kl}},$$

the real, symmetrical matrices that can be associated with the reflections of the individual space axes are commutative for integer spin, anticommutative for half-integer spin, and thus the symmetry of the matrix

$$R_s = e^{\pi i S_{12}} R_3$$

is given by

$$R_s{}^T = e^{-2\pi i S_{33}} R_s = (-1)^{2S} R_s;$$

the space reflection matrix is symmetrical for integral S, antisymmetrical for half-integral S. Hence, in any theory where I appears as a symmetrical matrix that commutes with R_s, the symmetry properties of B and R_s must coincide—which is the connection between spin and statistics. This situation is assured for a theory so constructed that the Lagrange functions of the individual fields are invariant under space reflection (I commutes with R_s) and make no explicit reference to internal, charge degrees of freedom (the invariant matrix I is not antisymmetrical). It should also be realized that the restriction to matrices I that commute with R_s is not necessarily a physical assumption but can be a choice of representation. Thus, for half-integral spin fields, the transformation

$$\chi \rightarrow e^{\pi i/4 R_{st}} \chi$$

converts $R_s I$ into $R_s\, iR_{st} I$, where $R_{st} I$ also commutes with all $S_{\mu\nu}$ but reverses the commutation properties of I relative to R_s.

Finally, it should be acknowledged that all these remarks concerned with the connection between spin and statistics have counterparts in the earlier work of W. Pauli.

[1] J. Schwinger, these PROCEEDINGS, **44**, 223, 1958.

[2] Some examples of the systematic use of this generator to derive field commutation properties are presented in "Differential Equations of Quantum Field Theory" *Lectures at Stanford University* (1956).

THE RELATION BETWEEN LONGEVITY AND OBESITY IN HUMAN BEINGS

BY LINUS PAULING

GATES AND CRELLIN LABORATORIES OF CHEMISTRY,* CALIFORNIA INSTITUTE OF TECHNOLOGY, PASADENA, CALIFORNIA

Read before the Academy, April 28, 1958

During recent years there has developed an increased interest in the question of the dependence of life span on various factors, such as weight, sex, country versus city dwelling, lipoprotein concentration in blood plasma, cigarette smoking, and exposure to high-energy radiation, and much statistical information about it has been gathered.[1] Some effort has also been made, in the case of factors, such as radiation exposure, that can assume a series of values, to express by an equation the relation between the average life span of populations differing with respect to this factor and the parameter representing the factor. Usually a linear equation has been used, often a single term, corresponding to proportionality between the decrease in expected life span and the parameter.

Sometimes a linear term plus a negative constant has been used, corresponding to a threshold value of the parameter, below which the linear expression is not valid. It is, of course, not to be expected that the function would have a discontinuous slope, and a better function can easily be formulated.

The average life span $L(x)$ of populations differing with respect to the parameter x and random with respect to other factors may be expressed as a power series in x

$$L(x) = \sum_n l_n x^n. \quad (1)$$

In some cases the constant term (normal life span) and the linear term provide a satisfactory representation of the available information. An example is the decrease in life span due to increased incidence of leukemia following exposure to ionizing radiation, as discussed by Lewis.[2] As more information becomes available, it may be found that the linear function is not a satisfactory approximation and that further terms are needed.

Moreover, the first two terms of equation (1) provide an unsatisfactory representation of the function for large values of x, in that the life span becomes negative. In order that the simple function be valid for large as well as for small values of the parameter, the reciprocal of the life span or some other suitable function of it may be used. A convenient quantity, equivalent to the reciprocal of the life span, is the relative increase in longevity accompanying change of the parameter to zero, defined as

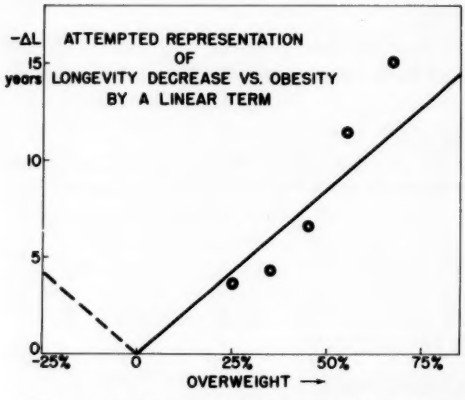


FIG. 1.—The best straight-line approximation to the five experimental points and passing through the assumed normal weight is shown. The dashed line indicates decreased longevity due to emaciation.

$$\Lambda(x) = \frac{L_0 - L(x)}{L(x)}, \quad (2)$$

with $L_0 = L(0)$. Λ becomes infinite as $L(x)$ becomes zero. In the case of a factor that is applied at some time in life, rather than beginning at birth, L may be taken as the life expectancy at that time rather than the life span. $\Lambda(x)$ can be represented by a power series:

$$\Lambda(x) = \sum_n \lambda_n x^n. \quad (3)$$

There are some factors affecting longevity for which the power-series expression must include the quadratic term. An example is the dependence of longevity on body weight. Both obesity and emaciation decrease the life span. There is, accordingly, a body weight (related to height and build) that gives the maximum life span. If the parameter x is the fractional deviation from this optimal body weight the linear term $l_1 x$ or $\lambda_1 x$ vanishes, and the simplest approximate expression relating decrease in longevity to body weight is the quadratic term $l_2 x^2$ or $\lambda_2 x^2$. Because of the presumed difference in nature of action of obesity and emaciation, a cubic term would be expected to be required for a good approximation.

Statistical information about the decrease in life span in relation to obesity has been published by Dublin and Marks.[3] The reported average decrease in life-span

is 3.6 years for 25 per cent overweight; 4.3 for 35 per cent; 6.6 for 45 per cent; 11.4 for 55 per cent; and 15.1 for 67 per cent. These values have been described[1] as 0.17 year average effect of 1 per cent overweight, and the linear function has been assumed by other authors to be valid even for 1 ounce (0.04 per cent) of overweight.[4]

The five points and assumed linear function are shown in Figure 1. The approximation is rather poor, the mean deviation being 1.8 years. Moreover, as mentioned above, the function is an unsatisfactory one, in that there is a discontinuity in the slope at normal weight. A better approximation is two straight lines—one, with value zero, up to 18 per cent overweight, and the other, with slope about 0.31 year per per cent overweight, above 18 per cent. This function, too, is unsatisfactory because of the discontinuity in its derivative.

The best representation by a quadratic function with minimum at the assumed normal weight is shown in Figure 2. A somewhat better representation is given by a general quadratic function. The two functions are 36 w^2 and 27.7 $(w + 0.07)^2$, respectively, where w is the fraction over normal weight. The mean deviations for these two functions are 0.7 and 0.6 years, respectively.

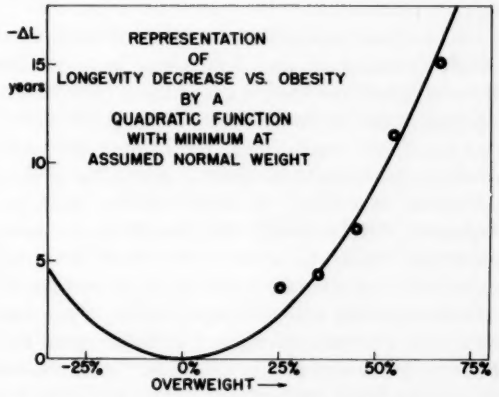


FIG. 2.—The best representation of longevity decrease by a quadratic function with minimum at assumed normal weight. The mean deviation for this function is one-third that for the best linear function.

The indication that a body weight 0.93 times that taken as normal maximizes longevity is unreliable. If equation (3) is used, rather than equation (1), a still better approximation is obtained (mean deviation 0.4 year) with no significant difference between optimal weight and normal weight. The best function is $\Lambda = 42.0\ (w + 0.003)^2$.

The idea of 1 ounce of overweight (0.04 per cent) is, of course, a ludicrous one, inasmuch as the optimal weight is uncertain by some pounds, and it would not be justified to mention it had it not been introduced into a serious discussion. The effect predicted for 1 ounce overweight by the erroneous linear function is about 1,000 times greater than that predicted by the quadratic functions.

However, there is some significance to the question of the predicted decrease in longevity for 10 pounds of overweight. The decrease predicted by our treatment is about 50 days, as compared with a little over 1 year given by the linear function. The new interpretation of the evidence about longevity and obesity may diminish somewhat the anxiety of individuals who are a few pounds overweight.

* Contribution No. 2333.

[1] H. B. Jones, *Proc. Health Physics Soc.*, p. 114, 1956.

[2] E. B. Lewis, *Science*, **125**, 465, 1957.

[3] L. I. Dublin and H. H. Marks, *Mortality among Insured Overweights in Recent Years* (New York: Metropolitan Life Ins. Co., 1952).

[4] E. Teller and A. Latter, *Life*, **44**, 64(1958); *Our Nuclear Future*, (New York: Criterion Books, Inc., 1958).

INTERRACIAL HYBRIDIZATION AND BREAKDOWN OF COADAPTED GENE COMPLEXES IN DROSOPHILA PAULISTORUM AND DROSOPHILA WILLISTONI

BY THEODOSIUS DOBZHANSKY AND OLGA PAVLOVSKY*

DEPARTMENT OF ZOÖLOGY, COLUMBIA UNIVERSITY, NEW YORK CITY

Communicated April, 1958

Certain local populations, or races, of the three sibling species, *Drosophila tropicalis, D. paulistorum*, and *D. willistoni* possess an interesting genetic structure.[1, 2, 3] More than half the individuals in these populations are heterozygotes for the same inverted section in one of their chromosomes, and fewer than half are homozygous. In a panmictic population at equilibrium, such a state of affairs is possible only if greater proportions of the homozygotes than of the heterozygotes are removed by a differential mortality. Natural selection then establishes a situation known as "balanced polymorphism"; the Mendelian population in which it occurs may possess a high fitnees, since the hybrid vigor (heterosis) in the heterozygotes compensates for the low adaptive value of the homozygotes.[4]

In *D. tropicalis*, a population in which 70 per cent of the individuals were heterozygous for a certain inversion, was encountered at Lancetilla, Honduras; elsewhere in Central America and in the West Indies the same chromosomal inversion had frequencies below 50 per cent, while in South American populations it was rare or altogether absent.[1, 3] In *D. paulistorum*, the sample from Urubamba, Peru, contained significantly more than 50 per cent of heterozygotes, while in two other localities on the eastern slope of the Andes in Peru the heterozygotes amounted to less than 50 per cent.[3] We have no evidence to show whether in these species the excesses of the heterozygotes are widespread or occur only in some small populations of isolated localities. In *D. willistoni* the situation is a little clearer. In at least three localities in northeastern Brazil the population samples contained more than 50 per cent of heterozygotes for a certain inversion (J, in the third chromosome), and in one of these localities samples taken on two successive years both showed this condition.[3] Elsewhere in South and Central America this chromosomal inversion is heterozygous in 50 or less per cent of the individuals, but another inversion (E, in the right limb of the second chromosome) reaches frequencies higher than 50 per cent of the heterozygotes in the Brazilian state of Ceará.[3]

The experiments reported below were designed to elucidate the nature of the genetic difference between populations in which the incidence of the heterozygotes is above and below 50 per cent. Our working hypothesis has been that this difference is quantitative rather than qualitative. Under balanced polymorphism, the incidence of the homo- and heterozygotes in a population at equilibrium is deter-

mined by their relative adaptive values. Furthermore, the superior fitness of the heterozygotes may be of different origins. If greater proportions of homozygotes than of heterozygotes are eliminated by differential mortality, there will be observed in the population a disturbance of the genotypic ratios demanded by the binomial square rule; on the other hand, the high Darwinian fitness of the heterozygotes may be due to their greater fecundity or a stronger sexual drive, in which case the Hardy-Weinberg ratios will remain unmodified.

Drosophila tropicalis.—As described previously,[1] the population of this species from Lancetilla, Honduras, contained 70.3 ± 5.3 per cent of heterozygotes for a certain chromosomal inversion. This incidence of the heterozygotes was observed among the larvae in the progenies of wild flies. These progenies were used to set up an experimental population in a "population cage." About 4 months later (corresponding, roughly, to six fly generations at the temperature of 25° C., at which all the experimental populations were kept), 90.7 ± 2.2 per cent of the larvae and 96.0 ± 1.4 per cent of the adult flies in the experimental population were inversion heterozygotes. The heterozygotes were evidently heterotic both in the natural and in the experimental environments, and their survival rates were higher than those of the homozygotes. Unfortunately, no experimental populations were made with flies from localities in which the incidence of the heterozygotes in the natural populations was below 50 per cent.

Drosophila paulistorum.—In the progenies of 29 wild females collected in the Urubamba Valley, Peru, 79.3 ± 7.6 per cent of the larvae were heterozygous for a certain inversion. In the progenies of 78 females from Tingo Maria, Peru, only 33.3 ± 5.3 per cent were heterozygous for the same inversion.[3] The distance between the two localities is roughly 400 km.

On February 6, 1956, a population cage, No. 165, was started with a foundation stock of about 4,045 flies, derived about equally from the 29 strains of Urubamba origin. On February 16 of the same year, experimental population No. 167 was started with about 4,220 flies derived from 66 strains of Tingo Maria origin. In late January, 1957, samples of the eggs deposited by the flies in the population cages were taken on several successive days, and larvae which hatched from these eggs were allowed to develop under optimal conditions. Examination of the chromosomes in the salivary glands of these larvae disclosed the following situation:

	Urubamba, No. 165		Tingo Maria, No. 167	
Larvae	Heterozygotes	Homozygotes	Heterozygotes	Homozygotes
Female	55	51	28	75
Male	52	42	28	69
Total	107	93	56	144

In the experimental population of Urubamba origin, 53.5 ± 3.5 per cent of the larvae were heterozygous for the inversion. This is significantly lower than the corresponding figure for the natural population (70.3 ± 7.6), but higher than that for the experimental population of Tingo Maria origin, 28.0 ± 3.2 per cent. The natural and the experimental populations from Tingo Maria appear to be alike in the incidence of the heterozygotes. The cause of the drop in the frequency of heterozygotes in the Urubamba experimental population is unclear. It is possible that one of the two homokaryotypes which must be present in these populations is less handicapped in relation to the other in the experimental than in the natural

environments. *Drosophila paulistorum* is a difficult material for cytological study, and we were unable to determine the incidence of the two kinds of the inversion homozygotes. However that may be, the Urubamba population is clearly different genetically from that from Tingo Maria, and this permits another interesting experiment to be made.

On December 12 and 19, 1956, population cages Nos. 170 and 171 were started with about 1,000 flies in each as the foundation stocks. In No. 170, the females (virgins) were derived from the Tingo Maria cage, No. 167, and males from the Urubamba cage, No. 165. In No. 171, the reciprocal cross was made. The populations of hybrid racial origin were kept for over 10 months, breeding freely; in late October, 1957, egg samples were taken in the population cages, and the chromosomes were examined in the larvae grown from these samples. The following conditions were found:

	TINGO MARIA × URUBAMBA NO. 170		URUBAMBA × TINGO MARIA NO. 171	
LARVAE	Heterozygotes	Homozygotes	Heterozygotes	Homozygotes
Female	6	102	2	104
Male	4	88	3	91
Total	10	190	5	195

A precipitate drop in the frequency of the inversion heterozygotes has evidently occurred in the experimental populations of hybrid origin. The populations have become virtually homozygous for one of the two gene arrangements which were present in both Urubamba and Tingo Maria populations. Hardly more than 15 or 16 fly generations have lived in the hybrid populations during the time interval between the start and the taking of the samples. Assuming that the initial frequency of the inversion heterozygotes was some 41 per cent (an average between the Urubamba and Tingo Maria populations, see above), the final frequency of about 4 per cent (averaging populations Nos. 170 and 171) indicates an intense selection in favor of one of the homozygotes.

Drosophila willistoni.—The experiments with this species have yielded results qualitatively similar to those described above for *D. paulistorum*. However, since *D. willistoni* is the more favorable of the two species for cytological study, the analysis of the results can be carried further.

The starting material consisted of 51 strains derived from flies collected at Recife, Pernambuco, Brazil, and 49 strains collected at Guaramiranga, Ceará, Brazil, in April, 1956. These localities are about 600 km. apart. Two inversions are of interest in these populations; inversion J in the third chromosome had the incidence of 60.7 ± 4.4 per cent in the Recife population and 54.9 ± 5.2 in that from Guaramiranga; inversion E in the right limb of the second chromosome had the incidence of 16.4 ± 3.3 per cent at Recife and 64.8 ± 5.0 at Guaramiranga.[3] On August 29 and 30, 1956, two experimental populations, Nos. 168 and 169, were started; the foundation stock in No. 168 consisted of about 3,250 flies from the 51 Recife strains, and in No. 169 of about 2,990 flies from the 49 Guaramiranga strains.

Some $4^1/_2$ months later, between January 14 and January 24, 1957, egg samples were taken in the populations, and the chromosomes were examined in the salivary glands of the larvae. The results were as follows (the sexes of the larvae not recorded):

	RECIFE, NO. 168		GUARAMIRANGA, NO. 169	
	Heterozygotes	Homozygotes	Heterozygotes	Homozygotes
Inversion III—J	123	77	85	115
Inversion II R—E	60	140	107	93

It can be seen that the frequency of heterozygotes for inversion J is the same in the natural and in the experimental population of Recife origin but that it has declined slightly in the experimental population of Guaramiranga origin; the frequency of heterozygotes for inversion E has risen in the experimental population of Recife origin, and perhaps declined in that of Guaramiranga origin. The examination of the experimental populations was repeated in late October to early November, 1957, when these populations were some 14 months old from the start. An attempt was made to distinguish not only the inversion heterozygotes from the homozygotes but also the two kinds of the homozygotes. For this purpose, adult flies which hatched in the food containers in the population cages were outcrossed, in individual cultures, to flies from a strain known to be homozygous for the "standard" gene arrangement in both the second and the third chromosomes. From each outcross, nine larvae were taken, and their salivary glands were stained with acetic orceine. Examination of the preparations disclosed whether the flies tested were hetero- or homozygous for the gene arrangements in their second and third chromosomes and, if they were homozygous, whether they carried the "standard" gene arrangement (denoted by a capital letter) or that modified by an inversion (denoted by a small letter). In the Recife population (No. 168), only the third chromosomes were recorded, and the following frequencies of the heterozygotes (Jj) and of the two kinds of homozygotes (JJ and jj) were found:

Recife, No. 168	Jj	JJ	jj
Adult females	64	28	8
Adult males	55	33	12
Total	119	61	20

The frequency of the Jj heterozygotes among the adult flies (59.5 per cent) is obviously close to that among the larvae in the same experimental population (61.5 per cent) and that in the natural population (60.7 per cent, see above). The data show, however, that one of the homozygous classes (JJ) is about three times as common as the other (jj). The observed frequencies of the three karyotypes differ appreciably from those demanded by the binomial square rule, as follows:

Recife, No. 168	Jj	JJ	jj
Observed	119	61	20
Expected	95.8	72.6	31.6
Difference	+23.2	−11.6	−11.6

Some differential mortality favoring the heterozygotes over the homozygotes evidently takes place in the Recife population. In the experimental population of Guaramiranga origin, both the second and the third chromosomes were recorded, with the following results:

Guaramiranga No. 169	Second Chromosome			Third Chromosome		
	Ee	EE	ee	Jj	JJ	jj
Adult females	60	28	12	51	39	10
Adult males	59	21	20	56	34	10
Total	119	49	32	107	73	20

The frequencies of the heterozygotes Ee and Jj among the adult flies from the experimental population of Guaramiranga origin are 59.5 and 53.5 per cent, respectively, which compares with the values 64.8 and 54.9 per cent found in the natural population of that locality (see above). This is a good agreement. However, as in the Recife population, we find the two classes of the homozygotes very

unequal in frequencies, and the proportions of the classes deviate from those expected if the Hardy-Weinberg equilibrium were realized.

GUARAMIRANGA No. 169	SECOND CHROMOSOME			THIRD CHROMOSOME		
	Ee	EE	ee	Jj	JJ	jj
Observed	119	49	32	107	73	20
Expected	99.3	58.9	41.9	121.0	80.0	27.0
Difference	+19.7	−9.9	−9.9	+14.0	−7.0	−7.0

In the Guaramiranga population, as in that from Recife, there evidently is some differential mortality which favors the heterozygotes and discriminates against the homozygotes. However, hybridization of these populations shows that they differ in genetic structure. Two experimental populations, Nos. 178 and 179, were started on February 20, 1957, each with a foundation stock of about 1,000 flies, among which one sex was derived from Guaramiranga and the other from Recife. In No. 178 the cross was Guaramiranga (No. 169) ♀♀ × Recife (No. 168) ♂♂, and in No. 179 the reciprocal. About 10 months later, in December, 1957, egg samples were taken, and the proportions of the hetero- and homozygotes were determined in the larvae which grew from them. The results were as follows:

POPULATION No.	SECOND CHROMOSOMES		THIRD CHROMOSOMES	
	Heterozygotes	Homozygotes	Heterozygotes	Homozygotes
178, larvae	55	145	90	110
179, larvae	68	132	76	124
Total	123	277	166	234
Per cent	30.75	69.25	41.5	58.5

The frequency of heterozygotes for the inversion J in the third chromosome is significantly lower in the hybrid populations than in the parental Recife population and equal to or lower than that in the Guaramiranga population. The inversion E heterozygotes are less frequent in the hybrid populations than at Guaramiranga, and about as frequent as in the experimental population of Recife origin. A further check on this decline in the frequencies of the heterozygotes in the hybrid populations is provided by an analysis of the zygotic constitution of the flies in the hybrid populations, made in March, 1958. The determinations were made by outcrossing the adult flies from the population cages to a standard strain in individual cultures and examining the chromosomes in up to 9 larvae in each progeny. The following results were obtained:

POPULATION No.	SECOND CHROMOSOME			THIRD CHROMOSOME		
	Ee	EE	ee	Jj	JJ	jj
178, adult ♀♀	15	20	5	17	15	8
178, adult ♂♂	12	25	3	11	28	1
179, adult ♀♀	15	22	3	11	26	3
179, adult ♂♂	12	26	2	13	26	1

In both populations combined, the frequency of adults heterozygous for the second chromosome inversion, Ee, is now 33.8 per cent, which agrees well with the figure 30.75 per cent found some months earlier among the larvae from the same populations. The corresponding figure for the third chromosome inversion heterozygotes are 32.5 and 41.5 per cent, i.e., the heterozygotes seem to be even less frequent among the adults than among the larvae (since the larval and the adult samples were not taken simultaneously, this frequency difference cannot be considered established).

A comparison of the observed frequencies of the homo- and heterozygotes in

populations Nos. 178 and 179 with those expected according to the Hardy-Weinberg rule shows the following:

Population Nos. 178 and 179	Second Chromosome			Third Chromosome		
	Ee	EE	ee	Jj	JJ	jj
Observed	54	93	13	52	95	13
Expected	60.0	90.0	10.0	59.0	91.5	9.5
Difference	−6.0	+3.0	+3.0	−7.0	+3.5	+3.5

The chi-squares are 1.17 for the second chromosome and 2.25 for the third; the observed values agree well with the expected ones. This is in contrast with the situation in populations of geographically pure Recife and Guaramiranga origin (Nos. 168 and 169, see above), in which significant excesses of heterozygotes were found. Wallace[5] has recently emphasized that the adaptive values of genotypes cannot be safely inferred from data on relative frequencies of homo- and heterozygotes in populations not at equilibrium. This stricture does not apply to our data, since we examined the experimental populations when they were more than a year old, at which time such populations usually approach an equilibrium status fairly closely. The differential mortality which favored the heterozygotes in populations of geographically pure origin is not observed in those of geographically mixed origin. It does not necessarily follow that in the latter the heterozygotes have no adaptive advantage, but, if they have such an advantage, it resides in traits other than differential survival in preadult stages. These populations are being continued, and we hope to report on their further behavior in due time.

Discussion.—Classical genetics liked to think of the genotypes of populations, races, and species as aggregates of genes with more or less autonomous effects, each gene selected on its own merits as adaptive in a given environment. The fact that adaptively coherent genotypes are, at least in higher organisms, integrated systems of genes is slowly gaining recognition. Studies on natural populations of *Drosophila* have supplied some of the clearest evidence. Many populations are polymorphic for variant gene arrangements which differ in inversions of blocks of genes in some of their chromosomes. The polymorphism is usually balanced, owing to the inversion heterozygotes being heterotic over most of the range of the environments which the population normally encounters. It has, however, been shown[4] that the fitness of an inversion heterozygote or a homozygote depends upon the geographic origin of the chromosomes which it carries. As a rule, heterozygotes whose chromosomes are derived from the same population are highly fit; heterozygotes which carry chromosomes of different geographic origins may or may not be. The rapid decline in the frequencies of the heterozygotes in the hybrid populations of *D. paulistorum* described above is a striking demonstration of this fact.

The heterosis is not a necessary consequence of being heterozygous for chromosomes with a given pair of gene arrangements; it is determined by the gene complexes which these chromosomes contain. Natural selection acts to adjust mutually, or coadapt, the gene contents of the chromosomes found in a given Mendelian population to yield high fitness in heterozygotes. The adaptive importance of the inversions, which enable us to distinguish under the microscope the chromosomes with the different gene complexes, is to guard the integrity of these complexes by means of suppression of the crossing over between the chromosomes which carry them. There is no selection pressure toward coadaptation of the

chromosomes found in different geographically separate and non-interbreeding populations. The evidence reported in the present article indicates that the chromosomal inversions present in the original populations produced heterosis in the natural as well as in the experimental environments. The behavior of the experimental populations of hybrid origins shows, however, that cytologically similar chromosomes were not identical in the gene contents in different populations.

Wallace[5] set forth an ingenious hypothesis, that variant gene arrangements maintained by natural selection in the same population are likely to be such that the integrity of the coadapted gene complexes will be protected from disintegration by crossing over. He pointed out the rarity in natural populations of so-called triads of versions, in which blocks of genes may be exchanged by crossing over in heterozygotes which carry certain combinations of the chromosomes. The populations of *Chironomus* studied by Acton[6] are in accord with Wallace's rule; rules of this kind are, however, not expected to hold except in a statistical sense; and Levitan and Carson[7] stressed the existence in *D. robusta* of situations not in accord with it. Although our experiments were not concerned with triads of inversions, their results have a bearing on Wallace's basic assumption. In the experimental populations of geographically uniform origins, heterosis is maintained, since the gene complexes coadapted in the natural population by natural selection continue to be protected by the inversion heterozygosis from breaking up. However, in the experimental populations of geographically mixed origins, there are two kinds of chromosomes with identical gene arrangements but containing different constellations of genes. Crossing over between such chromosomes yields new gene combinations, most or all of which never existed in nature because the geographic isolation of the parental populations prevented their hybridization. The evidence shows unambiguously that the recombination weakens or even destroys the heterosis conditioned in the geographically uniform populations by the coadapted gene complexes.

The agreement between our results and those of Vetukhiv[8] should be noted. This investigator found that hybridization of populations of *D. pseudoobscura* from different geographic regions results in a transitory heterosis in the F_1 and in a more or less pronounced breakdown of fitness in the F_2 generation. Some species of *Drosophila* are evidently differentiated geographically into local populations or races, which, although they are quite similar in the externally visible morphology, are distinct enough genetically that gene exchange between them leads to a loss of fitness. This conclusion evidently cannot be generalized to apply to races in other organisms, and particularly not to human races; it cannot legitimately be used to give aid or comfort to racism or to racists.

Summary.—The incidence of individuals heterozygous for certain chromosomal inversions exceeds 50 per cent in some local populations or races of *D. tropicalis*, *D. paulistorum*, and *D. willistoni*. In other populations of the same species the frequencies of the same inversion heterozygotes are below 50 per cent. Experimental populations made with flies of a given geographic origin generally retain the characteristics of the local race from which the founders of the population were derived. The chromosomal polymorphism is maintained in the populations because the inversion heterozygotes are superior in fitness to the homozygotes.

Experimental populations were set up, the founders of which were hybrids be-

tween races having more and fewer than 50 per cent of heterozygotes for certain inversions. In these experimental populations of mixed geographic origins the frequencies of the heterozygotes fell to or below the frequency levels in the parental races having fewest inversions. The superior fitness of the inversion heterozygotes depends upon coadaptation of the gene complexes which the chromosomes carry. In hybrid populations these gene complexes are broken up by crossing over, with a consequent loss of the heterosis.

* The work reported in this article has been carried out under Contract No. At-(30-1)-1151, United States Atomic Energy Commission.

[1] Th. Dobzhansky and O. Pavlovsky, these PROCEEDINGS, **41**, 289–95, 1955.

[2] A. B. da Cunha, *Evolution*, **10**, 231–34, 1956.

[3] C. Pavan, Th. Dobzhansky, and A. B. da Cunha, these PROCEEDINGS, **43**, 226–34, 1957.

[4] Th. Dobzhansky, *Genetics*, **33**, 588–602, 1948, and **35**, 288–302, 1950; *Atti IX Congr. Internaz. Genetica*, pp. 435–49, 1954; *Cold Spring Harbor Symposia Quant. Biol.*, **22**, 385–93, 1957.

[5] B. Wallace, *Am. Naturalist*, **87**, 343–58, 1953; *Evolution*, **12**, 113–15, 1958.

[6] A. B. Acton, *Am. Naturalist*, **92**, 57–58, 1958.

[7] M. Levitan, H. L. Carson, and H. D. Stalker, *Am. Naturalist*, **88**, 113–14.

[8] M. Vetukhiv, these PROCEEDINGS, **39**, 30–34, 1953; *Evolution*, **8**, 241–51, 1954, **10**, 139–46, and **11**, 348–60, 1957.

INFORMATION TO CONTRIBUTORS

THE PROCEEDINGS is the official organ of the NATIONAL ACADEMY OF SCIENCES and of the NATIONAL RESEARCH COUNCIL for the publication of brief accounts of important current researches of members of the ACADEMY and of the COUNCIL and of other American investigators. The PROCEEDINGS will aim especially to secure prompt publication of original announcements of discoveries and wide circulation of the results of American research among investigators in other countries and in all branches of science.

ARTICLES should be brief. The viewpoint should be comprehensive in giving the relation of the paper to previous publications of the author or of others and in exhibiting, where practicable, the significance of the work for other branches of science. Elaborate technical details of the work and long tables of data should be avoided, but authors should be precise in making clear the new results and should give some record of the methods and data upon which they are based.

MANUSCRIPTS should be prepared with a current number of the PROCEEDINGS as a model in matters of form, and should be typewritten in duplicate with double spacing, the author retaining one copy. Illustrations should be confined to text figures of simple character, though more elaborate illustration may be allowed in special instances to authors willing to pay for their preparation and insertion. Particular attention should be given to arranging tabular matter in a simple and concise manner.

MANUSCRIPTS will be accepted only from members of the Academy who will assume responsibility for the propriety and scientific standards of the paper and for any printing costs in excess of those allowed them by the Academy. Manuscripts should be addressed to *Proceedings of the* NATIONAL ACADEMY OF SCIENCES, 2101 Constitution Ave., Washington 25, D. C.

GALLEY PROOF will be sent, with the understanding that charges for author's corrections shall be billed to him. Author should therefore make final revisions on the typewritten manuscripts. Original artwork or figures cannot be returned unless a request is made when the manuscript is submitted. Page proofs will not be sent.

REPRINTS should be ordered when proof is returned, on the reprint order form which will be inclosed with the proof. The order blank gives a tabular estimation of the cost of reprints; a copy will be supplied at any time upon request.

CONTENTS

	Page
ASTRONOMY.—A THEOREM ON FORCE-FREE MAGNETIC FIELDS . . . . . *By L. Woltjer*	480
BIOCHEMISTRY.—THE EFFECTS OF HYDROXYLAMINE ON THE $C^{14}O_2$ FIXATION PATTERN DURING PHOTOSYNTHESIS . . . . . *By J. A. Bassham, M. Kirk, and M. Calvin*	491
BIOCHEMISTRY.—PROTEIN BIOSYNTHESIS BY A CELL-FREE BACTERIAL SYSTEM . . . . . *By Mirko Beljanski and Severo Ochoa*	494
BIOCHEMISTRY.—VITAMIN K, AN ESSENTIAL FACTOR FOR THE PHOTOCHEMICAL ACTIVITY OF ISOLATED CHLOROPLASTS . . . . . *By Norman I. Bishop*	501
BIOCHEMISTRY.—IONIZATION OF INDIVIDUAL GROUPS IN DIBASIC ACIDS, WITH APPLICATION TO THE AMINO AND HYDROXYL GROUPS OF TYROSINE . . . . . *By John T. Edsall, R. Bruce Martin, and Barbara K. Hollingworth*	505
BIOCHEMISTRY.—PTERIDINES IN PHOTOSYNTHESIS—AN ARTIFACT OF PAPER CHROMATOGRAPHY . . . . . *By R. C. Fuller, I. C. Anderson, and H. A. Nathan*	518
BIOCHEMISTRY.—ON THE EMISSION OF TRYPTOPHAN . . . . . *By Irvin Isenberg and Albert Szent-Györgyi*	519
BIOCHEMISTRY.—ON DISSOCIATION AND RECOMBINATION OF HUMAN ADULT HEMOGLOBINS A, S, AND C . . . . . *By Harvey A. Itano and S. J. Singer*	522
BIOCHEMISTRY.—EFFECT OF AGE ON THE ENZYME ACTIVITY IN ERYTHROCYTES . . . . . *By Paul A. Marks, Anne B. Johnson, and Erich Hirschberg*	529
BIOCHEMISTRY.—SPONTANEOUS NUCLEATION IN SUPER-SATURATED VAPORS . . . . . *By Worth H. Rodebush and John V. Clarke, Jr.*	536
BIOCHEMISTRY.—STUDIES ON THE EXCITED STATES OF PROTEINS . . . . . *By Richard H. Steele and Albert Szent-Györgyi*	540
BOTANY.—WALL THICKENING IN SIEVE ELEMENTS . . . . . *By Katherine Esau and Vernon I. Cheadle*	546
GENETICS.—INFLUENCE OF THE NUCLEOLUS ON MITOSIS AS REVEALED BY ULTRAVIOLET MICROBEAM IRRADIATION . . . . . *By Mary Esther Gaulden and Robert P. Perry*	553
GENETICS.—THE IMMUNE STATUS OF LONG-TERM SURVIVORS OF LETHALLY X-IRRADIATED MICE PROTECTED WITH ISOLOGOUS, HOMOLOGOUS, OR HETEROLOGOUS BONE MARROW. . . . . *By N. Gengozian, T. Makinodan, C. C. Congdon, and R. D. Owen*	560
GENETICS.—GENETIC STUDIES OF IRRADIATED NATURAL POPULATIONS OF DROSOPHILA. II. 1957 TESTS . . . . . *By Wilson S. Stone and Florence D. Wilson*	565
MATHEMATICS.—A NEW PROOF OF THE STRONG MARKOV THEOREM OF CHUNG. . . . . *By D. G. Austin*	575
MATHEMATICS.—DYNAMIC PROGRAMMING, SUCCESSIVE APPROXIMATION, AND MONOTONE CONVERGENCE . . . . . *By Richard Bellman*	578
MATHEMATICS.—ON THE THEOREM OF BERTINI FOR LOCAL DOMAINS . *By Wei-Liang Chow*	580
MATHEMATICS.—THE STRUCTURE OF PRIME RINGS WITH MAXIMUM CONDITIONS . . . . . *By A. W. Goldie*	584
MATHEMATICS.—ON THE STRUCTURE OF COMPACT RIEMANNIAN MANIFOLDS . . . . . *By W. Klingenberg*	586
MATHEMATICS.—A FORMULA FOR THE MULTIPLICITY OF A WEIGHT . *By Bertram Kostant*	588
PATHOLOGY AND BACTERIOLOGY.—TRANSDUCTION BY BACTERIOPHAGE P1: ABNORMAL PHAGE FUNCTION OF THE TRANSDUCING PARTICLES. *By Jane N. Adams and S. E. Luria*	590
PATHOLOGY AND BACTERIOLOGY.—THE FUNCTION OF EXTRACELLULAR ENZYMES OF DUTCH ELM DISEASE PATHOGEN . . . . . *By Akhtar Husain and A. E. Dimond*	594
PATHOLOGY AND BACTERIOLOGY.—NOMENCLATURE OF THE ACTINOMYCINS . . . . . *By Selman A. Waksman, Edward Katz, and Leo C. Vining*	602
PHYSICS.—ON THE NATURE OF FREE RADICALS DETECTED BY PARAMAGNETIC RESONANCE IN BIOLOGICAL SUBSTANCES . . . . . *By Ichiro Miyagawa, Walter Gordy, Norimitsu Watabe, and Karl M. Wilbur*	613
PHYSICS.—ADDENDUM TO SPIN, STATISTICS, AND THE TCP THEOREM . *By Julian Schwinger*	617
PHYSIOLOGY.—THE RELATION BETWEEN LONGEVITY AND OBESITY IN HUMAN BEINGS. . . . . *By Linus Pauling*	619
ZOÖLOGY.—INTERRACIAL HYBRIDIZATION AND BREAKDOWN OF COADAPTED GENE COMPLEXES IN DROSOPHILA WILLISTONI . . *By Theodosius Dobzhansky and Olga Pavlovsky*	622